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As isoformas do receptor P2X7 e o neuroblastoma: o papel crucial da isoforma B na quimiorresistência

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Tese apresentada ao Instituto de Química da Universidade de São Paulo para obtenção do Título de Doutor em Ciências Biológicas (Bioquímica)

Orientador: Profª Drª Claudiana Lameu

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A meus pais, grandes inspiradores, incentivadores e apoiadores de todas as minhas trajetórias.

A meu marido, companheiro e porto seguro.

À minha irmã, parceira e amiga de toda uma vida.

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"Quaisquer que sejam suas virtudes imaginadas, a fé é verdadeiramente inimiga da ciência aberta e honesta. Permanecer aberto aos poderes da conversa – a novas evidências e melhores argumentos – não é apenas essencial para a racionalidade. É essencial para o amor."

Sam Harris

RESUMO

O uso de agentes citotóxicos é a base de grande parte dos tratamentos oncológicos, e a quimiorresistência é o desafio central para sua efetividade. Mesmo as alternativas terapêuticas mais recentes, embora representem importantes contribuições, não são capazes de contornar ou combater a seleção natural das células resistentes ou restringir sua ampla capacidade adaptativa. No neuroblastoma, a recorrência tumoral ocorre em até 50% dos pacientes de alto risco. Desta forma, a identificação de novos alvos terapêuticos capazes de modular a sobrevivência ou morte celular em resposta aos tratamentos farmacológicos é crucial para a evolução da terapia anticâncer. Neste trabalho, investigamos o papel do receptor P2X7 na quimiorresistência e elucidamos as contribuições de suas isoformas A e B. Demonstramos que a isoforma B favorece a resistência aos fármacos estudados, por diversos mecanismos, incluindo o efluxo de fármacos via bombas do tipo MRP, resistência a retinoides, retenção das células em fenótipo indiferenciado, supressão da autofagia e indução de EMT, enquanto a isoforma A desempenha funções opostas e/ou complementares.

Palavras-chave: receptor P2X7; isoforma P2X7B; quimiorresistência; câncer infantil; células-tronco tumorais; transição epitélio-mesenquimal.

ABSTRACT

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Drug resistance is a major challenge for all oncological treatments that involve the use of cytotoxic agents. Recent therapeutic alternatives cannot circumvent the ability of cancer cells to adapt or alter the natural selection of resistant cells, so the problem persists. In neuroblastoma, recurrence can occur in 50% of high-risk patients. Therefore, the identification of novel therapeutic targets capable of modulating survival or death following classical antitumor interventions is crucial to address this problem. In this study, we investigated the role of the P2X7 receptor in chemoresistance. Here, we elucidated the contributions of P2X7 receptor A and B isoforms to neuroblastoma chemoresistance, demonstrating that the B isoform favors resistance through a combination of mechanisms including drug efflux via MRP-type transporters, resistance to retinoids, retaining cells in a stem-like phenotype, suppression of autophagy, and EMT induction, while the A isoform has opposite and/or complementary roles.

Keywords: P2X7 receptor; P2X7B isoform; chemoresistance, childhood cancer; cancer stem cells; epithelial-mesenchymal transition.

LIST OF ABBREVIATIONS AND ACRONYMS

ADP	Adenosine Diphosphate
ADRN	Adrenergic
ATP	Adenosine Triphosphate
BBG	Brilliant Blue G
BCRP	Breast Cancer Resistance Protein
CSC	Cancer Stem Cell
DBH	Dopamine Beta Hydroxylase
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
iPSC	Induced Pluripotent Stem Cell
MES	Mesenchymal
MET	Mesenchymal-Epithelial Transition
MRP1	Multidrug Resistance Protein 1
NBD	Nucleotide Binding Domain
P-gp	P-glycoprotein
PHOX2A	Paired-Like Homeobox 2A
PHOX2B	Paired-Like Homeobox 2B
PI	Propidium Iodide
RQ	Relative Quantification
RT-q-PCR	Reverse-Transcription Quantitative Polimerase Chain Reaction
SE	Standard Error
shRNA	Small Hairpin RNA
TGF-β	Transforming Growth Factor β
TMD	Transmembrane Binding Domain
TME	Tumor Microenvironment

UDP	Uridine Diphosphate
UTP	Uridine Trophosphate
VEGF	Vascular Endothelial Growth Factor

1. General Introduction:

1.1. Cancer, the classic problem

According to data from 2018, cancers are the second leading global cause of death, accounting for 1 in 6 deaths worldwide (WHO 2018). Originating from the uncontrolled proliferation of mutated cells, cancers have a diverse and complex biology, challenging scientists and health professionals for decades. Likewise, their etiology is diverse and multifactorial, encompassing environmental and genetic factors, in addition to aging, whose importance varies according to tumor type.

In summary, cancers are proliferative lesions that evolve into malignant tumors. Their pathology was summarized very elegantly in the so-called "hallmarks of cancer", proposed in 2000, reformulated in 2011 and ultimately reviewed in 2022, which consist of a set of changes necessary for tumorigenesis and tumor progression (Hanahan and Weinberg 2011; Hanahan 2022). As fundamental characteristics for the accumulation of these changes, genetic instability, which leads to mutations, and inflammation promoted by the tumor itself have been highlighted (Darwiche 2020).

The classical therapeutic approach is based on cytotoxic treatments – chemo- and radiotherapy – and surgical interventions. Such approaches were effectively able to treat countless patients, extending their life expectancy. However, their limitations have become progressively evident (Zugazagoitia et al. 2016). As early as the 2000s, a plateau of similar efficacy for different chemotherapy drugs was observed (Schiller et al. 2002).

Various advances have been made in the oncology field, such as the advent of immunotherapy. Over the years, the sum of efforts has effectively led to a decrease in

cancer-attributable deaths, especially in high-income countries (Hulvat 2020). Metastasis and drug resistance, however, persist as the main challenges responsible for the aggressiveness of high-risk diseases capable of inducing resistance and tumor recurrence. Thus, cancer remains the second leading cause of mortality worldwide.

1.2. Neuroblastoma

In young people and children (0 to 19 years old), cancer corresponds to the main global cause of death, the most preeminent ones being leukemias, brain tumors, lymphomas, and solid tumors, including neuroblastoma and Wilms tumor. New diagnoses worldwide reach 300,000 children per year (WHO 2018).

Survivors of childhood tumors have a high degree of morbidities throughout their lives: among those who respond to treatment and reach adulthood, 60 to 90% have chronic diseases (Hulvat 2020). These include subsequent neoplasms, in addition to persistent effects of chemotherapy and radiotherapy.

Neuroblastoma is a solid and extracranial tumor that affects portions of the sympathetic peripheral nervous system, mainly adrenal glands and paravertebral ganglia, and is the most prevalent tumor type in children under 5 years of age. Evidence points out that the tumor arises in neural crest cells committed to the sympathetic-adrenal lineage, which will later be distributed to sympathetic tissues. As such, neuroblastoma is a tumor of embryonic origin (Maris et al. 2007). Recently, Schwann cell precursors have been shown to also give rise to chromaffin cells, which produce catecholamines that integrate the adrenal medulla (Furlan et al. 2017), raising the possibility of a diverse embryonic origin for neuroblastoma (Delloye-Bourgeois and Castellani 2019).

In support of the heterogeneity of neuroblastoma tumors, the cells that constitute the tumors were classically divided into two lineages: N-type cells, neuroblastic, with neuronal adrenergic properties; S-type, nonneuronal Schwann-like cells, similar to substrate-adherent melanocytes; or type I, intermediate. This classification continues to be improved. More recent studies have classified neuroblastoma cells into two types, determined by the activation of different superenhancers by panels of specific transcription factors, leading to different gene expression profiles and different molecular markers: MES-like (mesenchymal) cells, poorly differentiated and presenting mesenchymal features, identified by CD133 expression; and ADRN-like (adrenergic) cells, with a higher degree of differentiation and sympathetic noradrenergic characteristics, expressing the PHOX2A, PHOX2B and DBH genes (Van Groningen et al. 2017). The prevalence and regulation of such phenotypes is related to tumor aggressiveness.

Neuroblastoma cases are stratified according to risk classification, which is related to the type of genomic alteration observed, the patient's age and, of course, the prognosis. High-risk patients are typically diagnosed after 18 months of age and have segmental chromosomal abnormalities and MYCN amplification, and their long-term survival rate is less than 50%. The most common metastatic sites are bone and bone marrow (National Cancer Institute - NIH). In these cases, treatments may include chemotherapy, radiation therapy, surgery, myeloablative therapy followed by autologous hematopoietic stem cell transplantation, and treatment with isotretinoin to induce cell differentiation and/or immunotherapy, achieving a 5-year survival rate of up to 62% (National Cancer Institute - NIH).

1.3. Chemotherapy agents, drug resistance and main mechanisms

Antineoplastic chemotherapeutics are drugs capable of acting preferentially on tumor cells, reducing their ability to survive or multiply (Bhoopathi et al. 2021). The selectivity of tumor cells over healthy cells is based almost exclusively on replicating activity, which is markedly high in most tumor cells. However, the tumor mass is known to be heterogeneous, which leads to two problems: first, not all tumor cells are affected by the treatment; and second, damage to healthy cells and tissues occurs (American Cancer Society).

Classic chemotherapy agents have different mechanisms of action, but all of them culminate in deleterious effects for the survival or multiplication of tumor cells. They are categorized as DNA alkylating agents, antimetabolites, antibiotics, topoisomerase inhibitors, antimitotics, or corticosteroids (American Cancer Society).

In addition to the activity of anticancer agents, chemoresistance occurs through several mechanisms, either intrinsic or acquired. When intrinsic, they are genetically determined, with selection of resistant populations occurring when the treatment is administered. When acquired, cells respond to environmental pressures by triggering adaptive processes. The most common resistance mechanisms are evasion of apoptosis, repair of damage caused to DNA or other targets by the chemotherapy agent, cell cycle escape and quiescence, drug efflux or inactivation mechanisms, and induction of changes in the pharmacological target, among others (Longley and Johnston 2005).

In the heterogeneous tumor mass, there are cells of different lineages. In addition to the tumor cells themselves, stromal cells make up the microenvironment: immune cells, fibroblasts, mesenchymal and endothelial cells (Bremnes et al. 2011; Arnaud-

Sampaio et al. 2020a). Among the tumor cells, variations also occur, related to the phenotype and degree of differentiation.

Cancer stem cells (CSCs) correspond to a population of tumor cells that retain the capacity for self-renewal and differentiation, which can lead to tumor recurrence by allowing the regeneration of the different cell types that constitute the tumor mass. Authors differ between the hypothesis that they would originate from a reserve of stem cells from the healthy tissue, or that they would result from the "dedifferentiation" of tumor cells. In the second case, the stem-like phenotype would not be stable and could be reversed or reacquired according to signals received from the tumor microenvironment (Ratajczak et al. 2010; Tang 2012).

CSCs have a high proliferative capacity, but they are also able to remain dormant or quiescent, replicating very slowly or remaining static for a long time, making them invisible to the action of anticancer drugs. Such a state of quiescence is reversible, and the reactivation of these cells can occur many years after the primary tumor has remitted. Although it is often described in less differentiated cells, dormancy is not a specific attribute of undifferentiated cells or stem cells (Phan and Croucher 2020).

A phenomenon to which is attributed a great contribution to cellular plasticity and tumor heterogeneity is the epithelial-mesenchymal transition (EMT). EMT consists of the acquisition of mesenchymal characteristics by cells whose initial phenotype is predominantly epithelial. However, it is important to emphasize that the product of this process is not necessarily typical mesenchymal cells but a spectrum of cells with phenotypes more or less similar to that of typical mesenchymal cells (Pal et al. 2021).

Epithelial cells have apical-basal polarity and are held together by intercellular junctions, constituting an organized tissue on the basement membrane, while

mesenchymal cells have an elongated morphology, are not connected by intercellular junctions and have greater mobility and ability to reorganize the extracellular matrix (Shibue and Weinberg 2017). EMT can be triggered by epigenetic mechanisms or gene expression regulation, such as signaling by transcription factors. It occurs, as well as its reverse process (MET, mesenchymal-epithelial transition), at different stages of embryogenesis, regulating phenotypes to allow the movement of cells and the constitution of new embryonic tissues, in addition to the healing of injured tissues. However, when appropriated by tumor cells, such mechanisms result in cells with greater plasticity and capacity to adapt to environmental stresses – such as the presence of chemotherapeutic drugs – plus greater mobility. Therefore, cells became more able to migrate and reorganize the extracellular matrix. Furthermore, EMT generates cells with a lower degree of differentiation, similar to CSCs (Smith and Macleod 2019).

CSCs show high expression of drug transporters capable of expelling drugs, such as ATP-binding cassette transporters (ABC transporters), also known as efflux pumps (Dean 2009). Such transporters expel drugs from the cells, preventing their intracellular effects.

Physiologically, ABC transporters are responsible for regulating intracellular levels of hormones, ions, lipids and other molecules, in addition to protecting cells from xenobiotics and exogenous agents, so the deficiency of some transporters is often related to diseases such as cystic fibrosis (Tarling et al. 2013). However, in tumor cells, their functions end up protecting tumor cells from drugs, conferring resistance.

ABC transporters are composed of four typical transmembrane domains, two of which are NBD (nucleotide binding domains) that hydrolyze ATP and two TMD

(transmembrane binding domains) that recognize the substrates to be transported (Dean et al. 2001). They are divided into 7 subfamilies, totaling 48 carriers. Among them, P-glycoprotein (P-gp, ABCB1), MRP1 (ABCC1) and BCRP (ABCG2), the first transporters characterized and whose preeminent clinical relevance remains supported by recent evidence, are of special interest (Robey et al. 2018).

The wide range of existing transporters relies on the heterogeneity of TMD domains, allowing the transport of the most diverse substrates. In fact, the ability to promote drug resistance is especially related to transporters with lower selectivity, which are capable of loading a wider range of substrates, and sometimes to high levels of expression of the transporters, since expression at low levels may not be enough to expel the molecules in a way that significantly interferes with their clinical effect (Robey et al. 2018).

In addition to the expression of transporters, cells have another mechanism to remove unnecessary or harmful components from cells: autophagy, also appropriated by tumor cells.

Autophagy is the process of cell self-degradation, consisting of the digestion of intracellular components that are either harmful or no longer useful. This process generates byproducts that can be used either as substrates for anabolism or for the generation of cellular energy (Uchiyama et al. 2008). Thus, it is an extremely relevant process in cellular plasticity scenarios and possibly in phenotypic changes related to dormancy state, CSC generation, and EMT. However, autophagy also triggers cell death, and its roles are highly relevant for the removal of senescent or neoplastic cells from tissues (Santana-Codina et al. 2017; D'Arcy 2019). In the context of antitumor drug resistance, autophagy can either contribute to the survival of tumor cells or

participate in the induction of death (Yonekawa and Thorburn 2013; Santana-Codina et al. 2017). Most evidence on different types of cancer and using diverse chemotherapy protocols point out that anticancer treatments usually induce autophagy and therefore lead to drug resistance (Smith and Macleod 2019).

In addition to providing substrates and energy to dormant cells, autophagy also participates in the turnover of transcription factors crucial for maintaining cell cycle suppression and pluripotency, in addition to preventing the transition to a permanently senescent state. Tumor cell dormancy is dependent on autophagy, both for the survival of disseminated cells in a quiescent state and for their reactivation, allowing the growth of previously dormant cells to establish a secondary tumor (Sosa et al. 2014; Vera-Ramirez et al. 2018).

Evidence demonstrates that CSCs are dependent on the autophagy process for the maintenance of the stemness state (Smith and Macleod 2019). The reprogramming of somatic cells to the pluripotent state, for example, generating cells known as iPSCs (induced pluripotent stem cells), depends on high levels of autophagy (Ma et al. 2015).

Environmental signals that lead to EMT, such as hypoxia and stimulation by TGF-β, also induce autophagy (Smith and Macleod 2019). Likewise, transcription factors related to the induction of pluripotency, such as SOX-2 and NANOG, are also related to autophagy, reducing mTOR activity (Wang et al. 2013).

1.4. The purinergic system

Purinergic signaling is the transmission of autocrine and paracrine intercellular signals mediated by nucleotides and nucleosides, either purinergic or pyrimidinergic. The main

signaling molecule of the purinergic system is extracellular ATP (eATP), in addition to adenosine, its dephosphorylated form (Burnstock 2007).

Purinergic receptors are ubiquitously expressed and are divided into three classes: P1 receptors, which are metabotropic and adenosine-responsive; P2X channels, which are ionotropic and ATP-responsive; and P2Y receptors, metabotropic and responsive to ATP, UTP, ADP, UDP and UDP-glucose (Burnstock 2007). The extracellular concentration of these molecules is finely regulated by several mechanisms, including the activity of nucleoside transporters, which control the release and uptake of nucleotides and nucleosides, and ectoenzymes, which control the degradation of nucleotides in the extracellular space.

1.5. The P2X7 receptor and its splicing variants

The P2X7 receptor (P2X7R) is a trimeric ionotropic receptor whose subunits are composed of a cytosolic N-terminal domain, two transmembrane segments separated by an extracellular loop, and a C-terminal tail, also cytosolic (MacKenzie et al. 1999). Its expression has already been confirmed in several cell types, such as macrophages, T cells, mast cells, microglia, epithelial cells and glial cells (Kopp et al. 2019).

Upon activation of this receptor by ATP, an ion channel permeable to small cations is opened, allowing the entry of Ca^{2+} and Na^+ and the exit of K^+, leading to downstream events responsible for the basal functions of the receptor (Di Virgilio et al. 2001). Among them are cell proliferation, promotion of inflammation, phagocytosis, and nociception. However, the sustained activation of the receptor leads to the opening of a non-selective pore that is permeable to molecules of up to 900 Da. This phenomenon triggers cell death, which is the first function ever described for the P2X7 receptor (Di Virgilio et al. 2001; Adinolfi et al. 2005).

In physiological situations, eATP concentrations are in the range of hundreds of nanomolars. However, situations that lead to the release or extravasation of ATP from the cell, as pathogenic or stressful stimuli, result in concentrations reaching hundreds of micromolars and culminating in the activation of the P2X7 receptor, the P2X channel that has the lowest binding affinity for ATP (Surprenant et al. 1996; North and Barnard 1997; Pellegatti et al. 2008; Conley et al. 2017).

Depending on the activation tone of the P2X7 receptor – that is, activation intensity and intermittence – the triggered effects vary (Baricordi et al. 1999; Adinolfi et al. 2005). These variations may also be attributed to the existence of different splicing variants, summing up 10 different isoforms in human cells, named P2X7A to P2X7J (Cheewatrakoolpong et al. 2005; Feng et al. 2006).

The human *p2rx7* gene is located in the chromosomal region 12q24.31 and has 13 exons (Zhou et al. 2009), which give rise to a protein of 595 amino acids (Buell et al. 1998). The transcriptional product that generates the complete protein corresponds to the P2X7A isoform, the best studied and known to open the macropore in the cell membrane, a function attributed to the C-terminal portion of the protein (Arnaud-Sampaio et al. 2020b). Retention of the intron located between exons 10 and 11 results in the retention of a stop codon, terminating translation prior to the formation of the complete C-terminal portion and generating a truncated protein, which is P2X7B. In addition to these isoforms, variants P2X7E, P2X7G and P2X7J are also truncated, lacking the C-terminal tail. Among them, P2X7B is the only one capable of constituting a functional ion channel (Cheewatrakoolpong et al. 2005; Giuliani et al. 2014; Arnaud-Sampaio et al. 2020b). Macropore opening, however, is not triggered in the absence

of the C-terminal tail, and as such, P2X7B does not trigger cell death or allow dye uptake, as occurs with P2X7A (Adinolfi et al. 2010; Arnaud-Sampaio et al. 2020b).

While the P2X7G and P2X7H isoforms are generated by the deletion of the TM1 transmembrane portion, the P2X7C and P2X7F isoforms are generated by changes in the extracellular loop. All four of these isoforms are not capable of constituting functional receptors. Finally, P2X7J, one of the truncated versions of the receptor, exerts a function opposite to the P2X7A isoform, protecting against cell death, a phenomenon called negative dominance (Cheewatrakoolpong et al. 2005; Feng et al. 2006; Arnaud-Sampaio et al. 2020b).

1.6. Implications of P2X7 in aspects of tumor progression

The great attention received by the P2X7 receptor as a possible therapeutic target for cancer treatment is based on some premises. First, there is evidence of high expression of this receptor in several tumor types (Adinolfi et al. 2002; Slater et al. 2004; Solini et al. 2008; Ryu et al. 2011). Additionally, the concentration of eATP, the endogenous P2X7 receptor agonist, is markedly elevated in tumors, reaching hundreds of micromolars (Pellegatti et al. 2008; Conley et al. 2017), a concentration capable of activating the P2X7 receptor (North and Barnard 1997).

The best-known functions of the P2X7 receptor are the triggering of cell death and the activation of inflammatory processes. First, researchers characterized the cell death triggered by cell lysis upon macropore opening, leading to necrosis. However, progressively, evidence has emerged demonstrating that processes related to apoptosis, such as membrane depolarization, activation of caspases, membrane blebbing, metabolic reprogramming and reorganization of signaling lipids on the cell surface, also occur following P2X7 receptor activation (Wiley and Dubyak 1989;

Spranzi et al. 1993; Di Virgilio 2021; Rabelo et al. 2021). Naturally, if P2X7 receptor agonism induces cell death and the receptor is widely expressed in tumor cells, it is reasonable to assume that the P2X7 receptor is a promising target for antitumor therapy.

In fact, this possibility has been investigated since the 1980s, and encouraging results have been achieved in several settings (Rapaport 1988; White et al. 2005; Shabbir et al. 2008). More recently, it has been shown that glioma cells with high levels of P2X7 receptor expression were more sensitive to radiotherapy and that expression levels could even be used as indicators of a better prognosis in radiotherapy treatment (Gehring et al. 2012, 2015). Likewise, the administration of ATP was also able to potentiate the cytotoxic action of temozolomide in glioblastoma cells, demonstrating a possible synergistic action with chemotherapy. Another study further linked the P2X7 receptor to ATP-induced cytotoxicity in glioblastoma cells (Tamajusuku et al. 2010; D'Alimonte et al. 2015).

It is important to note that in the tumor microenvironment, several cell types coexist. Tumor-infiltrated immune cells also show high levels of P2X7 expression and are therefore also affected by the high extracellular concentration of ATP in the tumor microenvironment (Arnaud-Sampaio et al. 2020a; Di Virgilio 2021).

The antitumor immune response is strongly influenced by the expression and activity of the P2X7 receptor, and its deletion or pharmacological inhibition has already been shown to impair the organization of an effective antitumor response on several occasions (Hofman et al. 2015; Arnaud-Sampaio et al. 2020b; Grassi and De Ponte Conti 2021). On the other hand, animals knocked out for the P2X7 receptor start to exhibit an immunosuppressive immune response and accelerated tumor progression

(Adinolfi et al. 2015; De Marchi et al. 2019). Thus, considerations about the expression and activity of the P2X7 receptor in tumors should always be aware of possible confounding factors related to their roles in different cell types.

Controversies began to arise when the first evidence of trophic effects emerged, including demonstrations that transfection of the receptor into HEK-293 cells, which do not naturally express P2X7, conferred advantages for cell growth, rather than favoring cytotoxicity (Baricordi et al. 1999; Adinolfi et al. 2005, 2012). Since then, numerous studies have been carried out, and the evidence was divided between pro- or antitumor roles of the receptor, finally leading to the understanding that the activation tone would be crucial for the determination of the triggered response (Di Virgilio 2021). In addition, while its expression in immune cells is generally favorable for an effective antitumor response, its overexpression in cancer cells is often related to trophic responses and tumor progression.

Recently, it was demonstrated that after irradiating glioblastoma cells, there was a switch of predominant isoforms in the surviving cells, with a pronounced reduction of P2X7A and a corresponding increase in P2X7B expression levels, associated with an increase in pluripotency markers and cellular senescence. Additionally, administering receptor antagonists after irradiation potentiated the cytotoxicity (Zanoni et al. 2022).

Corroborating these results, another study showed that the sensitivity of acute myeloid leukemia cells to daunorubicin is dependent on the expression of P2X7 receptor isoforms, with P2X7A being related to greater sensitivity to chemotherapy, while P2X7B is related to tumor recurrence and is positively modulated by the treatment (Pegoraro et al. 2020).

In fact, such findings are congruent with what is known about the P2X7B isoform: although it retains the functionality of the ion channel, this isoform does not allow the opening of the macropore related to cytotoxicity, so its trophic effects predominate (Adinolfi et al. 2010; Pegoraro et al. 2020).

Tumor progression depends on the suppression of mechanisms that lead tumor cells to death, in addition to favoring signaling pathways that promote their growth, survival and adaptation. Thus, the role of P2X7B seems especially suitable for such cells. In fact, studies in the oncology area support the hypothesis that P2X7B has predominantly pro-tumor roles (Pegoraro et al. 2021a).

In addition to the P2X7B isoform, the variant named nfP2X7, whose conformation is characterized by an unconventional folding of the protein, also corresponds to a nonfunctional isoform in terms of opening a macropore. The expression of nfP2X7 has been shown to be increased in the presence of high levels of eATP, similar to those seen in the tumor microenvironment (Gilbert et al. 2019). nfP2X7 is widely expressed in tumor sections of several types of cancer, and a specific antibody was able to reduce skin lesions in basal cell carcinoma, showing that this could be a promising therapeutic alternative (Gilbert et al. 2017) and reinforcing the hypothesis that variants incapable of triggering cytotoxicity even in the presence of high concentrations of eATP may play protumor roles.

In neuroblastoma, poorly studied regarding its relationship with the P2X7 receptor, the existing evidence points out that 1. the P2X7 receptor is highly expressed in neuroblastoma tumors, and this high expression is reproduced in neuroblastoma cell lines; 2. high expression levels are correlated with worse prognoses; 3. receptor activation leads to trophic responses; and 4. chronic receptor antagonism in a murine

model reduced tumor progression and metastasis (Raffaghello et al. 2006; Amoroso et al. 2015; Ulrich et al. 2018). Together, these observations suggest that, in the neuroblastoma tumors studied, the trophic functions of the receptor prevail over cytotoxic or antitumor immunity, and it is therefore reasonable to assume that the functions of P2X7B predominate. However, very little is known about P2X7 receptor isoforms in neuroblastoma, and no approaches focused on drug resistance have ever been pursued.

In this sense, there remains a clear gap in the understanding of how the controversial responses of the P2X7 receptor and its isoforms influence chemoresistance in neuroblastoma and how this knowledge could evolve toward realistic progress in therapeutic opportunities for childhood cancer.

1.7. Relevance and objectives

Considering all the above, the aim of this work was to investigate the relevance of different P2X7 receptor isoforms in neuroblastoma drug resistance and elucidate the involved mechanisms. In fact, this questioning is fundamental to move forward in the study of the P2X7 receptor as a viable therapeutic alternative. Although it has been demonstrated how promising this path can be, studies aiming to resolve and reconcile apparently contradictory but certainly complementary evidence are essential to move on. Understanding the effects of the P2X7 receptor on drug resistance would allow the exploration of such knowledge for therapeutic purposes, addressing the persistent needs that thousands of patients each year face in the fight against resistant cancers. Additionally, little is known about the specific aspects related to neuroblastoma tumors, a cancer of such importance in pediatric oncology.

In response to such questions, studies were carried out giving rise to the article "P2X7 receptor isoform B is a key drug resistance mediator for neuroblastoma" (Arnaud-Sampaio et al. 2022) reproduced in full, without changes, in the following chapter of this thesis.

In addition, two comprehensive reviews of the scientific literature were conducted on the roles of the P2X7 receptor in other important aspects of tumor biology, including metastasis and immune response, giving rise to articles 4.2.2 and 4.2.3 in the attachments of this thesis.

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2. P2X7 receptor isoform B is a key drug resistance mediator for neuroblastoma

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2.1. Introduction

Drug resistance is undoubtedly the greatest challenge in the fight against cancer (Vasan et al. 2019). Although new treatment options are progressively developed, few surviving cancer cells are enough to promote disease relapse, and the multiple factors that influence the ability of cancer cells to evade therapy are highly complex. In the case of neuroblastoma, a childhood tumor affecting cells of the sympathetic peripheral nervous system, nearly 50% of high-risk patients either relapse or do not respond to first-line therapy protocols (Zafar et al. 2021). Therefore, the investigation of molecular targets involved in cancer cell fate determination, which shift cells toward either death or survival, is crucial for advancing cancer research.

The P2X7 receptor, an ion channel of the P2X family of ATP-gated ionotropic purinergic receptors, is of particular interest given its long-known cell death-inducing properties (Lara et al. 2020). Thus, it has been previously hypothesized that P2X7 receptor stimulation in cancer cells could present a survival challenge, contributing to

successful cancer therapy. Indeed, P2X7 receptor upregulation in brain tumor cells is a good prognostic predictor for radiation therapy response, and P2X7 receptor agonists have been shown to potentialize the cytotoxic effect of anticancer agents (Gehring et al. 2012, 2015; D'Alimonte et al. 2015).

The cell death-promoting role of the P2X7 receptor depends on the formation of a large nonselective membrane pore that is permeable to molecules of up to 900 Da. Macropore opening is triggered by sustained receptor stimulation and leads both to altered membrane permeability, allowing the influx of large molecules, and to ATP efflux (Pellegatti et al. 2005), increasing the ATP concentration in the extracellular space.

However, very high concentrations of extracellular ATP (5-10 mM) are required to activate the cytotoxic activity of the P2X7 receptor, and although these concentrations are probably achieved following classical cancer therapies leading to necrosis, such as chemotherapy or radiotherapy, they are rarely present in the untreated tumor microenvironment (TME) due to the activity of ubiquitous ectonucleotidases (Lara et al. 2020; Kepp et al. 2021). Therefore, the P2X7 receptor is usually active only as an ion channel within the TME, and as such, it has been shown to promote cell growth, neovascularization, matrix degradation and metastasis in various solid and liquid tumor models, including neuroblastoma (Amoroso et al. 2012; Lara et al. 2020; De Marchi et al. 2021; Pegoraro et al. 2021b).

In addition to the biphasic gating of the P2X7 receptor ion channel, the existence of distinct splice variants of the p2rx7 gene also plays a role in determining distinct cellular responses (Lara et al. 2020). Among the isoforms generated by alternative splicing of the human p2rx7 gene, the A and B isoforms are the only well-characterized functional

ion channels and are ubiquitously expressed (Pegoraro et al. 2021a). While P2X7A corresponds to the full-length variant, P2X7B arises from the retention of an intron containing a stop codon, which shortens the protein length (Cheewatrakoolpong et al. 2005; Arnaud-Sampaio et al. 2020b). This truncated version lacks the C-terminal tail that has been described as crucial to macropore opening. However, the P2X7B variant is still functional, as it retains the capability of opening the ion channel, leading to downstream cellular effects (Di Virgilio et al. 2018; Arnaud-Sampaio et al. 2020b). In previous studies, the P2X7A variant has produced death-promoting antitumor effects, while the P2X7B variant has been implicated in the trophic, metastatic and resistance-prone properties of tumor cells (Adinolfi et al. 2010; Giuliani et al. 2014; Pegoraro et al. 2020). Furthermore, our group has observed that the malignancy of neuroblastoma cells is related to increased P2X7B expression (Ulrich et al. 2018).

Therefore, we aimed to resolve the differential contributions of P2X7 receptor splice variants to the drug resistance of neuroblastoma by further analyzing the mechanisms involved. Our results point to multifaceted drug resistance, comprising many mechanisms that together build up a consistent resistant phenotype. On the one hand, we demonstrated that P2X7A favors retinoid sensitivity to neuroblastoma cell differentiation and autophagy and downregulates efflux pumps. On the other hand, we showed the complementary role of the P2X7B isotype in suppressing autophagy, inducing drug efflux, and favoring EMT. The obtained results are highly relevant for explaining the dual role of the P2X7 receptor in tumor biology, paving the way for innovative therapies for chemoresistant tumors.

2.2. Materials and Methods

2.2.1. Cell lines

ACN human neuroblastoma cells derived from bone marrow metastasis (RRID:CVCL_1068) and HEK 293 cells (ATCC® CRL-1573™) were genetically modified as previously described (Adinolfi et al. 2010, 2012). ACN cells were silenced with shRNAs from OriGene™ as detailed in Table 1, resulting in three derived cell lines.

HEK 293 cells, which do not natively express P2X receptors, were transfected for P2X7 receptor isoform A or B expression, resulting in the following derived cell lines: HEK 293-A, expressing P2X7A; HEK 293-B, expressing P2X7B; and HEK 293-*mock*, not expressing any P2X7 receptor isoform. Procedures for obtaining transfection constructs are described in detail elsewhere (Adinolfi et al. 2010).

Table 1: Target sequences of shRNAs used for ACN cell silencing

	Target sequence (5'-3')	Predicted phenotype
shRNA1 c.n. TI202483	ACGTTTGCTTTGCTCTGGT GAGTGACAAG	P2X7A$^-$/B$^-$
shRNA2 c.n. TI202486	CATTAGGATGGTGAACCA GCAGCTACTAG	P2X7A$^-$/B$^+$
*scrambled s*hRNA c.n. TR30012	−	P2X7A$^+$/B$^+$

2.2.2. Cell culture

The culture medium for ACN cells consisted of RPMI 1460 medium supplemented with 10% bovine fetal serum, 1% NEAA 100X solution (Gibco™, c.n. 15140122), 100 U/mL penicillin, 100 mg/mL streptomycin, and the clone selection antibiotic puromycin at a 500 nM final concentration. Cells were kept at 37°C and 5% CO_2.

HEK 293 cells were cultivated in DMEM/F12 culture medium supplemented with 10% bovine fetal serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and specific clone selection antibiotics (G48 sulfate at 200 ng/ml for HEK 293-A and HEK 293-*mock* cells and hygromycin B at 100 µg/ml for HEK 293-B cells). Cells were kept at 37°C and 5% CO_2.

2.2.3. Cell line characterization

For cell line validation and characterization of P2X7 receptor isoform expression patterns, PCRs were performed.

For ACN cells, TaqMan™ real-time RT–PCR assays were conducted. After RNA extraction and cDNA synthesis, as described below in the specific section, Taqman™ reactions were prepared containing 1 µl of cDNA, 1X Taqman™ Fast Advanced Master Mix, Applied Biosystems™, 1.8 µM final concentration of primers, 0.5 µM of FAM-MGB probe, and 1X GAPDH predesigned Taqman™ assay Hs02758991_g1 VIC-MGB. Cycling conditions consisted of 2 min at 50°C, 2 min at 95°C, followed by 40 cycles of 1 s at 95°C plus 20 s at 60°C in the thermocycler StepOnePlus®, Life Technologies. For quantification, we used the calculation method $2^{-\Delta\Delta Ct}$, normalizing scrambled-transfected sample values to 1.

The probe sequences were as follows: P2X7A 5′ CACAGCGGCCAGACCG 3′ - 6FAM; P2X7B 5′ ACAAGCGCTGCGTTAGT 3′ - 6FAM.

The primers used were as follows: P2X7A Forward 5′ CGGCTCAACCCTCTCCTACT-3′; P2X7A Reverse 5′ GGAGTAAGTGTCGATGAGGAAGTC-3′; P2X7B Forward 5′GGAAAATGGTTTGGAGAAGGAAGTG-3′; and P2X7B Reverse 5′CGATGAGGAAGTCGATGAACACA-3′.

For HEK 293 cells, conventional reverse transcription PCRs were used. After RNA extraction and cDNA synthesis, as described below in the specific section, PCRs were prepared with 1.25 U Taq DNA polymerase, Invitrogen™ in 1X Buffer, 2 mM MgCl2, 0.2 mM dNTPs, forward and reverse primers at 0.5 µM each, and 1 µL of cDNA in a 25 µL reaction. After denaturation at 94°C for 2 min, the reaction proceeded with 40 cycles of 94°C for 1 min + 50°C for 1 min + 68°C for 2 min. Finally, a final cycle of 68°C for 10 min was conducted to guarantee the final extension of DNA strands.

The obtained products were subjected to electrophoresis in a 1.5% agarose gel prepared with TAE buffer. Samples were run at 80 V for 30 min, and images were obtained on a UV transilluminator.

The primers used were as follows: P2X7B Forward: 5' CCCATCGAGGCAGTGGA 3'; P2X7B Reverse: 5' TAAAGCATGGAAAAGAGAATCTC 3'; panP2X7 Forward: 5' AGATCGTGGAGAATGGAGTG 3'; panP2X7 Reverse: 5' TTCTCGTGGTGTAGTTGTGG 3'; GAPDH Forward: 5' CCTCTGACTTCAACAGCGAC 3'; GAPDH Reverse: 5' CATGACAAGGTGCGGCTCCC 3'.

2.2.4. Tumorsphere culture

For culturing cells as tumorspheres, cells were seeded in 0.2% F-127 pluronic acid-coated hydrophobic suspension dishes in defined DMEM High medium containing 20 ng/ml EGF, 20 ng/ml FGF and 1X N-2 supplement, Gibco™, for at least 96 h. For flow cytometry, tumorspheres were dissociated with 2 mM EDTA and vigorously pipetted.

2.2.5. Cell viability – alamarBlue®

For cell viability assessments, cells were seeded on 96-well plates with black borders and clear bottoms at a density of 10^4 cells per well with complete medium overnight. After adherence and 2 h of starvation in serum-depleted medium, the chemotherapy drugs vincristine (300 nM) or doxorubicin (100 µM) were added, combined or not with the treatments (described in Table 2 and detailed in each figure), in supplemented medium without clone selection antibiotics (unless specifically stated) for 48 h.

For comparison of culture conditions, cells were also subjected to 2 h of starvation in serum-depleted medium and then incubated with the tested culture medium. To observe the effects of nutrient availability on drug resistance, a culture condition gradient composed of four conditions was tested: EBSS buffer, EBSS buffer + 2 g/dL glucose, MEM-EBSS medium, and MEM-EBSS + 10% FBS. For autophagy induction through serum starvation, the culture conditions consisted of the control group cultured in RPMI 1640 10% FBS and the starved group cultured in RPMI 1640 0% FBS.

After specific interventions, the cells were incubated for a 2 h period with a 1:10 alamarBlue® solution prepared in culture medium protected from light at 37°C and 5% CO_2. Fluorescence readings were performed in FlexStation III, Molecular Devices™, at 530-560/590 nm excitation/emission wavelengths, as specified by the manufacturer.

Cell viability values were calculated as percentages, adopting a control group considered 100%.

When analyzing the influences of a specific drug (detailed in Table 2) or culture condition on the effects of vincristine or doxorubicin, calculations were performed as described. First, the cell viability values of the group treated with the additional drug alone were calculated considering the cell viability value of the untreated group of the same cell line as 100%. A one-tailed paired t test analysis determined whether the treatment alone was cytotoxic considering a confidence interval of 95%.

If $p>0.05$: Cell viability values of all groups in the analysis were calculated considering the cell viability of the untreated group as 100% and compared altogether using one- or two-way ANOVA variance analysis, according to experimental design.

If $p<0.05$, additional treatment was considered cytotoxic, and as such, groups that received the cytotoxic additional drug were normalized considering the cell viability values of the group treated exclusively with the cytotoxic drug as 100%, and related groups were compared for statistically relevant differences using t tests (when only two samples) or ANOVA, according to the experimental design.

Table 2: Details of pharmacologic treatments other than chemotherapy drugs

	Description	Concentration
ATP	P2X7 receptor agonist	1 mM
BzATP	P2X7 receptor agonist	100 µM
γSATP	P2X7 receptor agonist	100 µM
A438079	P2X7 receptor antagonist	10 µM
BBG	P2X7 receptor antagonist	300 nM
Retinoic Acid	Neural differentiation inducer	5 µM
TGF-β	EMT inducer	5 ng/ml
EGF	EMT inducer	50 ng/ml
Probenecid	MRP-type inhibitor	1 mM
Verapamil	Pgp inhibitor	5 µM
Ko143 hydrate	BCRP inhibitor	30 nM
Rapamycin	Autophagy inducer	200 nM

2.2.6. Cell death – propidium iodide (PI)

For cell death analysis, cells were seeded in 12-well plates at a density of 3×10^5 cells per well with complete medium and incubated overnight. After adherence and 2 h of starvation in serum-depleted medium, chemotherapy drugs were added, combined or not with the treatments detailed in Table 2, in supplemented medium without clone selection antibiotics (unless specifically stated) for 48 h. After detachment with 2 mM EDTA, cells were washed in PBS, passed through a 40 µm cell strainer, and stained

for 10 min with a 2 μg/ml PI solution in PBS at 4°C. The positive control consisted of a mixed cell sample incubated in 2 μg/ml PI + 0.1% Triton X-100 PBS solution, while the negative control was a mixed cell sample incubated in pure PBS.

Cell death rates were determined in an Attune™ Acoustic Focusing Cytometer, Life Technologies™, with minimal acquisition of 50,000 events per sample at 200 μL/min flow speed. The obtained data were analyzed in FlowJo software (BD Biosciences).

2.2.7. Dose–response curves

To characterize the response pattern of the employed cell lines to the chemotherapy drugs, cell viability drug-response curves were performed employing crescent drug concentrations in intervals of 0.5 log units: 0-100 μM for vincristine, 0-10 mM for doxorubicin, and 0-30 mM for cyclophosphamide. For cell death (PI staining) drug-response curves, the concentration ranges were 0-10 μM for vincristine, 0-100 μM for doxorubicin, and 0-10 mM for cyclophosphamide. IC50 and EC50 concentrations were calculated based on nonlinear regression analysis performed in GraphPad Prism 5™ software.

2.2.8. RNA extraction

For relative gene expression analysis, cells were seeded on 6-well plates at a density of 3 x 10^6 cells per well. After adherence and 2 h of starvation in serum-depleted medium, the respective treatments were applied in supplemented medium without clone selection antibiotics (unless specifically stated).

The cells were then washed with PBS, collected directly in TRIzol and frozen at -80°C until extraction with cold chloroform, precipitated in isopropanol and washed in 75% ethanol, as instructed by the manufacturer's protocol.

2.2.9. cDNA synthesis and relative expression analysis – RT–qPCR

Reverse transcription reactions using both oligoDT and random hexamers were performed after treating RNA samples with DNAse. We used a RevertAid® reverse Transcription Kit from Invitrogen (Thermo Fisher) to synthesize cDNA from 2 µg of purified RNA from each sample following the manufacturer's protocol. Cycling conditions consisted of 5 min at 65°C, followed by 10 min at 25°C, 42°C for 60 min and 70°C for 10 min.

Primer pairs with the sequences specified in Table 3 and SYBR Green Master Mix 2x reagent were incubated with 1 µl of 10x diluted cDNA synthesis product. The cycling conditions for amplification were 95°C for 1 min for denaturation, 40X 95°C for 30 s, and 60°C for 1 min, followed by melting curve analysis performed in 3°C increments. The thermocycler StepOnePlus®, Life Technologies, was used. For quantification, we used the calculation method $2^{-\Delta\Delta Ct}$, normalizing control sample values to 1. As an endogenous housekeeping control, primers targeting the EMC7 gene were employed.

2.2.10. Protein expression analysis – Flow Cytometry

Protein expression levels were quantified by flow cytometry. Cells were detached from culture plates with 2 mM EDTA, fixed in PFA 4% for a minimum of 1 h, and blocked/permeabilized in 4% FBS + 0.1% Triton X-100 PBS solution. An overnight incubation was then performed with primary antibodies (Abcam© ab129450, ab3380, ab32574, ab207612, and ab1316; Molecular Probes® L10382) at a 1:100 dilution in 1% FBS PBS, followed by staining with Alexa Fluor® 555 anti-mouse or Cy5® anti-rabbit secondary antibodies in 4% FBS + 0,1% Triton X-100 PBS solution for 15 min.

Cytometric measurements were performed in an Attune™ Acoustic Focusing Cytometer, Life Technologies™, with BL-2 or RL-1 filter settings (Alexa Fluor® 555 anti-mouse or Cy5® anti-rabbit, respectively). Fluorescent thresholds were determined by negative samples incubated with secondary antibody in FlowJo software (BD Biosciences).

Table 3: qPCR primer sequences

Target	Sequence (5'→ 3')
P-gp (ABCB1) Forward	CGTGGGGCAAGTCAGTTCA
P-gp(ABCB1) Reverse	TCCTTCCAATGTGTTCGGCA
MRP1 (ABCC1) Forward	ACTAGGAAGCAGCCGGTGAA
MRP1 (ABCC1) Reverse	CTTCTGTGGGGACTTGACGA
BCRP (ABCG2) Forward	TGAAAAGGATGTCTAAGCAGGGA
BCRP (ABCG2) Reverse	GCAGGCCCGTGGAACATAA
N-cadherin Forward	GCCCAAGACAAAGAGACCCA
N-cadherin Reverse	TCAACTTCTGCTGACTCCTTCA
Fibronectin Forward	TGGGCAACTCTGTCAACGAA
Fibronectin Reverse	CCACTCATCTCCAACGGCAT
Nanog Forward	AGAAAGAGGTCTCGTATTTGCTG
Nanog Reverse	ACACTCGGTGAAATCAGGGT
Twist2 Forward	GACAGCAGTGACATCGGACA
Twist2 Reverse	GACCCAGAAGAAAAATCCAAACAGA
Twist1 Forward	CCACTGAAAGGAAAGGCATCAC
Twist1 Reverse	TATGGTTTTGCAGGCCAGTT

2.2.11. Cell cycle analysis

Cells were detached from culture plates with 2 mM EDTA, washed in PBS, fixed in 70% ethanol for 1 h at 4°C, passed through a 40 µm cell strainer, blocked and permeabilized in 1% FBS + 0.1% Triton X-100 PBS solution for 30 min with agitation. Next, the cells were incubated with anti-ki67 Millipore® antibody (AB9260) in 1% FBS + 0,1% Triton X-100 PBS solution for 45 min with agitation and further incubated with Alexa Fluor® 555 anti-rabbit IgG antibody for 15 min. Finally, the cells were washed and incubated with RNAse A 50 µg/ml solution in 0.1% Triton X-100 + 0.5% Tween 20 PBS solution for 15 min. Populations were measured in an Attune™ Acoustic Focusing Cytometer, Life Technologies™, with minimal acquisition of 50,000 events per sample at 200 µL/min. The obtained data were analyzed using FlowJo software (BD Biosciences).

2.2.12. Efflux activity – Hoechst 33342® staining followed by image cytometry (TissueFAXS®)

For efflux activity assessments, cells were seeded on 96-well plates with black borders and clear bottoms at a density of 10^4 cells per well with complete medium overnight.

Efflux activity by ATP-binding cassette (ABC) transporters was measured using the dye Hoechst 33342 at a 100 ng/ml solution prepared in culture medium, followed by incubation for 30 min and fixation in 4% PFA. A propidium iodide solution (PI) at 3 µg/mL was used to stain cell nuclei. Plates were scanned in the TissueFAXS®, TissueGnostics, fluorescence microscope, and cytometric quantifications were performed by StrataQuest® software.

Detection of PI staining by the nuclei mask tool in StrataQuest® software allowed mapping of the cell nuclei area in the images. Next, the fluorescence intensity for Hoechst 33342 was measured in the determined area and visualized as dot plots. As a negative control, samples stained with PI only were employed, while a positive control was prepared by cell fixation with 4% PFA before Hoechst 33342 staining. Thus, the method identified low Hoechst 33342 staining populations that were able to efflux the dye.

2.2.13. Drug interaction analysis

For drug interaction analysis, we employed the Bliss Independence model followed by *t* tests comparing transformed values of predicted and observed survival fractions to determine the statistical relevance of the observations, as described elsewhere (Demidenko and Miller 2019).

2.2.14. Statistical analysis

For the cell viability and efflux assays, variances among groups were compared by paired *t* tests and one- or two-way ANOVA according to the experimental design followed by Bonferroni posttests, considering a 95% confidence interval. For relative expression analysis, one-way ANOVA was employed, followed by Bonferroni posttest, also considering the 95% confidence interval.

2.3. Results

2.3.1. The P2X7B isoform is implicated in drug resistance, while the P2X7A variant is related to cell death induction

To investigate the influence of the P2X7 receptor and its isoforms on drug resistance and to confirm the function of each isoform, we chose two cell lines: a human neuroblastoma cell line (ACN cells) and HEK 293 cells. When submitted to 48 h treatments with drugs commonly used for neuroblastoma treatment, ACN cells responded well to doxorubicin, i.e., presented a significant decrease in their viability upon treatment, as expected in chemotherapy, but not to cyclophosphamide – which is also expected as cyclophosphamide is a prodrug activated by hepatic metabolism – and had pronounced resistance to vincristine, reaching a plateau with a surviving ~50% population observed in both cell viability and cell death assays (Fig. 1A and 1B). Similarly, HEK 293 cells did not show significant death rates upon vincristine or cyclophosphamide treatment (Fig. 1C).

A shRNA-based silencing approach was used to selectively decrease the expression levels of P2X7 receptor isoforms. shRNA1, with a sequence designed to silence P2X7 receptor isoforms A and B, successfully decreased their expression levels, whereas shRNA2 selectively silenced the B isoform according to what was expected based on its sequence (Fig. S1A). HEK 293 cells, a cell type endogenously lacking P2X7 receptors (Morelli et al. 2003), were transfected with the coding sequence of the isoform of interest, as described in the Methods section, generating three cell line subtypes: *mock* cells (control), HEK 293-A cells (P2X7A overexpression), and HEK 293-B cells (P2X7B overexpression) (Fig. S1B).

In our experiments, shRNA1-silenced ACN cells were the most susceptible to both vincristine and doxorubicin treatments, presenting the highest decrease in cell viability values (reminiscent viable population of 43.87% ± SE 5.2 (shRNA1) vs. 76.08% ± SE 6.75 (scrambled), and 69.08% ± SE 7.65 (shRNA2), $p=0.0034$) (Fig. 1D). In contrast, shRNA2-silenced cells had the highest doxorubicin resistance (reminiscent viable population of 52.75 ± SE 11.37 (shRNA2) vs. 9.81 ± SE 2.76 (shRNA1), and 27.25 ± SE 4.72 (scrambled), $p=0.0007$), robustly demonstrated in several different culture conditions (Fig. 1D and S2A).

For HEK 293 cells, isoform expression was also relevant to the vincristine response but not to doxorubicin. P2X7A overexpression was related to the highest decrease in cell viability upon vincristine treatment, reinforcing the previously demonstrated cell death-promoting roles of this isoform (Fig. S2B).

Although these findings may sound controversial, they are indeed complementary. In the ACN neuroblastoma cell model, nonsilenced cells express the P2X7A isoform, but not exclusively: they also express P2X7B, and the trimeric receptor may occur in a heterogeneous composition, representing a scenario closer to reality. In the HEK 293 model, the isoforms occur alone and are overexpressed, isolating and highlighting the function of each isoform.

As P2X7 receptor activity in neuroblastoma cells is less evident than that observed in the HEK 293 cell overexpression model, we treated ACN cells with P2X7 receptor agonists and antagonists. Vincristine treatment alone significantly decreased the viability of shRNA1 cells only (69.95% ± SE 7.99 vs. 100% in the control group, $p \leq 0.05$). However, the combination of vincristine and ATP, the endogenous P2X7 receptor agonist, promoted a similar effect in the nonsilenced cells (73.2% ± SE 9.46

vs. 60.73% ± SE 13.72 in the presence of ATP) (Fig. 2A). This finding corroborates the role of the P2X7A isoform in promoting vincristine-induced death, as observed in HEK 293 cells overexpressing this isoform. However, ACN shRNA2 cells did not respond to vincristine either in the presence or absence of ATP, suggesting that the P2X7B receptor alone is related to a drug-resistant phenotype (Fig. 2

Figure 1. Dose–response curve of chemotherapy in cells differentially expressing the P2X7 receptor and its isoforms. Dose–response curves of vincristine (n=6), doxorubicin (n=3) and cyclophosphamide (n=3) in neuroblastoma cells. A) Viability assays and B) cell death assays performed in ACN cells containing scrambled shRNA. C) Drug-response curves of the chemotherapy drugs vincristine, doxorubicin and cyclophosphamide in HEK 293 cells obtained by PI staining quantification by flow cytometry. D) Cell viability measurements of ACN cells

expressing (scrambled), silenced for both A and isoforms (shRNA1) and silenced for B isoform (shRNA2) after treatment with vincristine or doxorubicin. Data normalized by the respective cell type untreated control (n≥3). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Increased doxorubicin resistance of nonsilenced or shRNA2-silenced cells was attenuated by antagonism with Brilliant Blue-G (BBG), while shRNA1-silenced cell drug sensitivity remained unchanged (Fig. 2B). Regarding vincristine resistance, shRNA2-silenced cells showed decreased resistance upon selective antagonism with A438079, an effect that was not observed in nonsilenced cells, indicating that in this context, drug resistance is related to the specifically assembled P2X7 receptor with the B isoform (Fig. 2C).
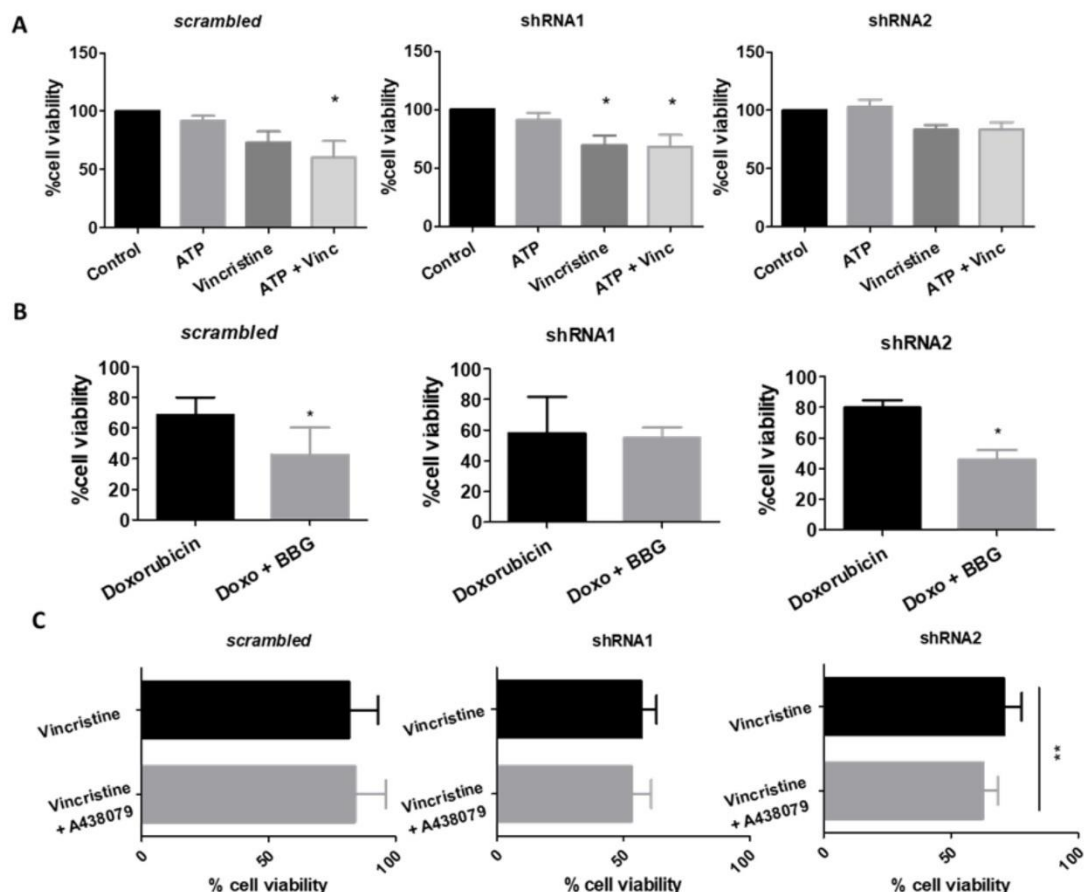
Figure 2. P2X7 receptor activity in response to chemotherapy. A) Cell viability measurements of ACN cells stimulated or not with ATP, the endogenous P2X7 receptor agonist, alone or in combination with vincristine (n≥3). B) Cell viability measurements of ACN cells stimulated with doxorubicin (doxo) in the presence or absence of the P2X7 receptor antagonist Brilliant Blue-

G (BBG) (n≥4). C) Cell viability measurements of ACN cells stimulated with vincristine in the presence or absence of the P2X7 receptor-specific antagonist A438079 (n≥4). Values were calculated as detailed in Methods section item 2.2.5. *$p<0.05$ and **$p<0.01$.

2.3.2. While the P2X7A isoform is critical for retinoid-induced neural differentiation of neuroblastoma cells, the P2X7B variant is related to EMT marker upregulation in HEK 293 cells and enhanced EMT-induced drug resistance of neuroblastoma cells

Because stemness and epithelial-mesenchymal transition (EMT) are closely related to chemoresistance 14, we characterized our cell lines, which differentially expressed P2X7 receptor isoforms with respect to the degree of differentiation and epithelial-mesenchymal phenotype transformation (Fig. 3). We assessed the mRNA transcript levels of vimentin and fibronectin, which are mesenchymal phenotype markers, and E-cadherin protein levels, which are indicative of the epithelial-like phenotype (Zeisberg and Neilson 2009). The mRNA levels of NANOG, a marker of neural progenitor cells (Vincent et al. 2017), were used as an indicator of stemness for neuroblastoma cells.

In ACN shRNA1-silenced cells, absence of the P2X7 receptor was related to increased expression of vimentin, fibronectin, and E-cadherin and decreased expression of NANOG (Fig. 3A), pointing to a mixed EMT-MET phenotype that cannot be restricted to a simple polarizing definition. The presence of the P2X7B isotype alone was related to decreased NANOG and fibronectin levels compared to nonsilenced cells, suggesting that these cells were not intrinsically more mesenchymal- or stem-like than those expressing both isoforms (Fig. 3A).

Treatment of ACN cells with BzATP, a stable P2X7 receptor agonist, increased E-cadherin levels in control cells, an effect that was not observed in either shRNA1- or shRNA2-silenced cells, suggesting an epithelial-prone P2X7A-related effect (Fig. 3B).

When treated with EGF and TGF-β, two known EMT-inducing factors, we observed that the lowest concentrations tested (50 ng/ml and 5 ng/ml, respectively) were optimal for inducing EMT in our model, producing the expected increase in vimentin expression levels. For this reason, we used these concentrations henceforth (Fig. 3C).

This increase in vimentin expression upon treatment with EMT-inducing growth factors matched the enrichment of P2X7 receptor expression (Fig. 3C) and increased cell quiescence (G0 state) in nonsilenced cells (Fig. 3D), culminating in decreased cell death with vincristine treatment (Fig. 3E).

Importantly, EMT made neuroblastoma cells more resistant to death induced by treatment with vincristine alone or in combination with BzATP, the P2X7 agonist. This finding suggests that the mesenchymal phenotype overcomes P2X7A receptor activation, which produces death-promoting antitumor effects that were observed in neuroblastoma cells expressing P2X7A and not treated with EMT-inducing growth factors (Fig. 2A).

Figure 3. P2X7 receptor isoforms in epithelial-mesenchymal transition and stemness of neuroblastoma cells. A) mRNA expression levels of vimentin, fibronectin and NANOG (n=3) and protein levels of E-cadherin (n=3) measured in scrambled, shRNA1 and shRNA2 cells. B) E-cadherin protein levels measured by flow cytometry in BzATP-treated vs. nontreated ACN cells (n=3). C) Vimentin and P2X7 receptor mRNA expression levels in scrambled ACN cells treated with combinations of low/high concentrations of TGF-β (5 or 25 ng/ml) and EGF (50 or 100 ng/ml) (n=3). D) Cell cycle phase distribution of scrambled ACN cells treated with EMT inducers at optimal concentrations (TGF-β 5 ng/ml + EGF 50 ng/ml) (n=3). E) Cell death measurements (PI-staining) of scrambled ACN cells treated with the P2X7 receptor agonist BzATP and/or vincristine, with or without treatment with EMT inducers at optimal concentrations (TGF-β 5 ng/ml + EGF 50 ng/ml) (n=3). $*p<0.05$; $**p<0.01$; $***p<0.001$.

HEK 293-A cells shifted the expression levels of Twist-2, a transcription factor indicative of EMT. This effect was prevented by ATP treatment, which was also observed in *mock* cells, demonstrating that this ATP-induced effect was not related to the P2X7 receptor. When HEK 293-B cells were induced to undergo EMT, N-cadherin levels increased, but Twist2 levels increased only with concomitant addition of ATP, suggesting that P2X7B favors EMT and that direct stimulation with ATP enhances this

effect, pushing cells toward a more mesenchymal phenotype. Because EMT did not occur in *mock* cells but only in P2X7B-overexpressing cells, we hypothesize that the acquisition of a mesenchymal phenotype is mediated by the B isoform, highlighting P2X7B as an EMT-favoring isoform (Fig. 4A).

When ACN neuroblastoma cells were EMT-induced and treated with either vincristine or doxorubicin, shRNA2 cells showed a significant increase in remaining cell viability (83.60% ± SE 8.79 vs. 70.95% ± SE 7.27 in nontreated cells, p=0.0443), an effect not observed in shRNA1 cells (Fig. 4B), suggesting that cells predominantly expressing the P2X7B isoform undergo EMT more effectively and thus become more drug-resistant.

When the expression of VEGF-A receptor, an EMT marker, was assessed, we found no differences among the untreated cell types, but this receptor was enriched in all groups when submitted to vincristine treatment, reinforcing the relevance of EMT in vincristine resistance of neuroblastoma cells (Fig. 4C).

Cancer cells cultured as tumorspheres are often used as a model for cancer stem cell enrichment in culture. Therefore, we compared neuroblastoma cells cultured in monolayers or as tumorspheres.

While shRNA1-silenced ACN cells cultured in monolayers responded better to vincristine when their counterparts were cultured as tumorspheres, isoform expression patterns were not relevant for drug responses, maintaining cell death rates within the same range for all cell types (Fig. 4D). This means that whatever advantages P2X7-silenced cells had for treatment sensitivity, they were suppressed in tumorspheres, and these cells become as resistant as their nonsilenced or shRNA2-silenced counterparts. Therefore, phenotype modulation promoted by tumorsphere culture compensated for

the absence of the P2X7 receptor. Thus, nonsilenced and shRNA2-silenced cells did not further increase drug resistance in the face of these stimuli.

shRNA1-silenced cells, when cultured as tumorspheres, reduced vimentin expression levels to 33.8% of the levels observed in the monolayer culture (RQ of 1.42 ± SE 0.2 (monolayer) vs. 0.48 ± SE 0.09 (tumorspheres), p=0.0021) (Fig. 4E), which may be interpreted as an indication of lower EMT grade. This may appear controversial to the fact that these cells respond poorly to vincristine in comparison to monolayer-cultured cells. In addition, both nonsilenced and shRNA1 cells had reduced fibronectin levels when cultured as tumorspheres (Fig. 4E). However, in our experiments, epithelial phenotype did not seem to be a good predictor of drug response, and EMT induction was only effective in enhancing drug resistance in shRNA2 cells, linking EMT-induced drug resistance to the P2X7B isoform.

Figure 4. Epithelial-mesenchymal transition and stemness of cells cultured in monolayers or as tumorspheres. A) mRNA expression levels of EMT-related transcription factors in HEK 293 cells, untreated, or treated with ATP and/or EMT inducers (EGF + TGF-β) (n=3). B) Cell viability measurements of vincristine- (n≥5) or doxorubicin-treated (n=4) ACN cells in the presence or absence of EMT inducers. Values were calculated as detailed in Methods section item 2.2.5. C) Expression levels of the VEGF-A receptor protein in ACN cells treated with or without vincristine. Measurements were performed by flow cytometry. (n=3) D) Cell death ratio of ACN cells cultured in monolayers or as tumorspheres upon treatment with vincristine. For normalization of cell death intrinsic to tumorsphere manipulation, values were calculated as detailed in Methods section item 2.2.5. E) mRNA expression levels of vimentin and fibronectin in ACN cells cultured in monolayers or as tumorspheres (n=3). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

To investigate neuroblastoma cell capability to differentiate, considering that the ACN cell line is poorly differentiated and mesenchymal-like per se (Van Groningen et al. 2017), we stimulated cells with retinoic acid, a neural differentiation inducer (Janesick et al. 2015). Retinoic acid treatment successfully decreased the expression levels of the neural progenitor marker NANOG in nonsilenced cells, as would be expected (RQ

1.42 ± SE 0.08 vs 0.48 ± SE 0.23 in retinoic acid-treated cells, p=0.0441). However, this reduction was not observed in shRNA1- and shRNA2-silenced cells; in fact, NANOG levels increased in shRNA1-silenced cells (RQ 0.41 ± SE 0.32 vs. 2.01 ± SE 0.69 in retinoic acid-treated cells, p<0.01) (Fig. 5A). This finding suggests that the absence of the P2X7A isoform prevented neural differentiation of ACN cells, maintaining cells in an undifferentiated phenotype.

Retinoic acid treatment increased the vincristine resistance of cells lacking the P2X7A variant (Fig. 5B), indicating that the absence of this isoform not only prevented neural differentiation but also enhanced resistance to vincristine, although not to doxorubicin (Fig. 5B).

Cellular quiescence is a dormant-like cellular state that allows cells to evade cancer therapy, which largely targets proliferating cells (Recasens and Munoz 2019). P2X7 receptor activation with γsATP, an ATP analog that is more resistant to hydrolysis, enriched quiescent populations of ACN cells and consequently decreased those in G1 phase (Fig. 5C and 5D). However, the presence of the selective P2X7 receptor antagonist A438079 reversed this effect in cells expressing the P2X7A isoform, while in shRNA1- and shRNA2-silenced cells, the effect was persistent (Fig. 5C and 5D), suggesting a P2X7A-related role.
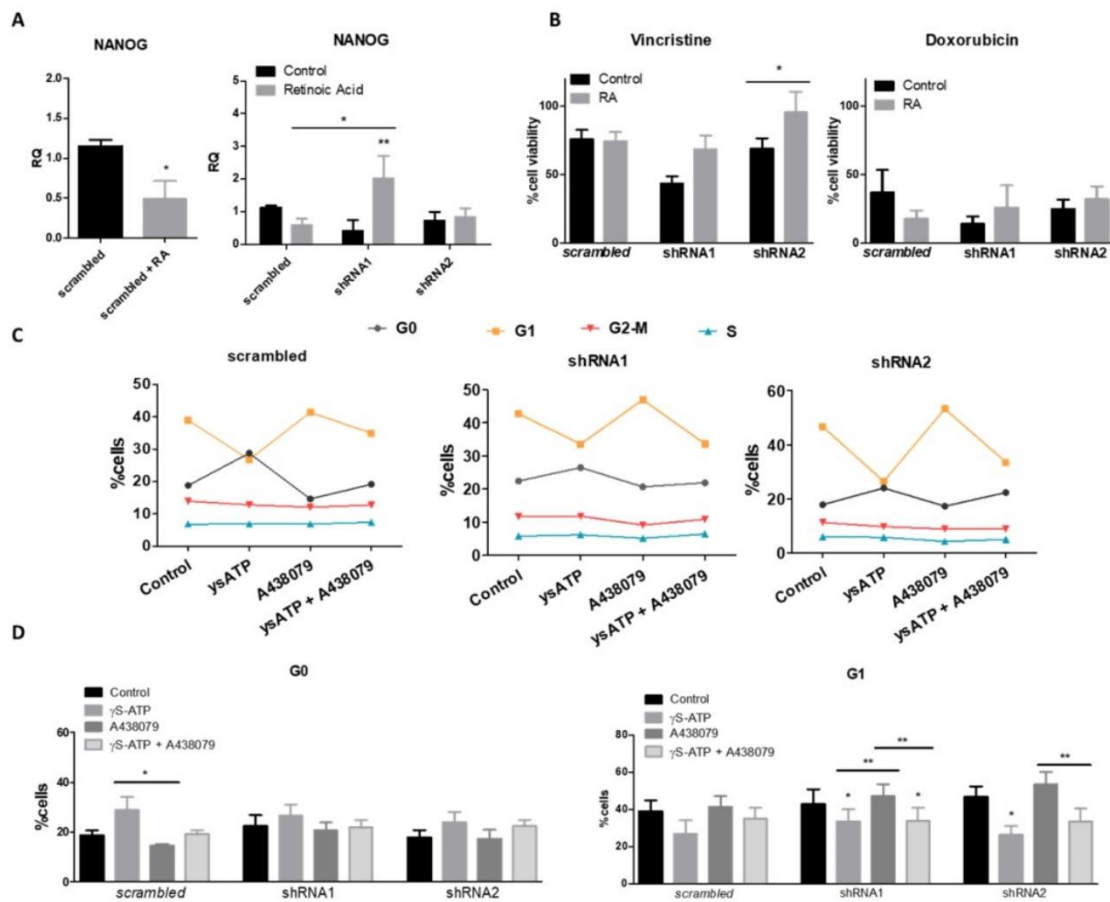
Figure 5. Retinoic acid-induced differentiation and cell cycle in neuroblastoma cells differentially expressing P2X7 receptor isoforms. A) NANOG mRNA expression levels in ACN cells treated with the neural differentiation inducer retinoic acid (RA) at a 5 μM concentration (n=4). B) Cell viability measurements of ACN cells treated with RA and/or vincristine or doxorubicin (n=3). Values were calculated as detailed in Methods section item 2.2.5. C) Complete representation of cell cycle phases of ACN cells after treatment with P2X7 receptor agonist and/or antagonist (n=5). D) Distribution of ACN cells in cell cycle phases G0 and G1 after treatments with ysATP (an ATP analog resistant to hydrolysis), A438079 (selective P2X7 receptor antagonist) or both (n=5). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

2.3.3. shRNA2-silenced cells exhibit decreased levels of autophagy markers, and its stimulation with rapamycin attenuates vincristine resistance, whereas starvation attenuates doxorubicin resistance.

When testing culture conditions, we observed that ACN neuroblastoma cells appeared to respond well to doxorubicin under most culture conditions, whereas vincristine resistance was severely impaired. The expression patterns of isoforms in ACN cells

appeared to determine both vincristine and doxorubicin responses (Fig. 6A). Nonsilenced cells responded effectively to vincristine in EBSS-glucose and MEM-EBSS but not MEM-EBSS + FBS, pointing to a detrimental role of serum supplementation for drug efficacy when both P2X7 receptor isoforms are present, possibly indicating that growth factors and other molecules present in serum depend on the P2X7 receptor to induce proliferation or enhance cell survival. In shRNA1-silenced cells, the drug response was better in MEM-EBSS and MEM-EBSS + FBS, reinforcing the hypothesis that the P2X7 receptor participates in the cellular response to serum compounds favoring drug resistance. Finally, shRNA2-silenced cells responded poorly under all conditions. For HEK 293 cells, culture conditions were the only determinant of doxorubicin effects (Fig. S2B).

These differences suggest that metabolic pathways play an extremely important role in determining the drug resistance phenotype of both ACN and HEK 293 cells.

HEK 293 cells overexpressing P2X7A presented the lowest serum-induced growth ratio, followed by P2X7B-overexpressing cells, both of which were lower than *mock* cells. This demonstrates that these cells can grow efficiently in the absence of serum, and as such, adding serum does not increase the growth efficiency as much (Fig. 6B). The ability to grow in serum absence is a malignancy-related ability, as it allows cell survival in stressful situations. This is consistent with the increased drug resistance observed in P2X7 receptor-expressing neuroblastoma cells, such as nonsilenced and shRNA2-silenced subtypes (Fig. 1E).

Autophagy is tightly regulated and related to nutrient supply and stress stimuli. Therefore, we hypothesized that this important metabolic phenomenon might be involved in the observed drug resistance. To investigate this possibility, we measured

autophagy markers in ACN cells and tested whether autophagy manipulation could modulate the drug response.

LC3 expression was decreased in shRNA2-silenced cells compared with the other cell groups, indicating low autophagic activity in cells that expressed the B isoform only. In addition, Beclin-1 levels increased in control and shRNA1-silenced cells treated with vincristine, but this shift was not observed in shRNA2-silenced cells (Fig. 6C and 6D). This finding suggests that shRNA2 cells do not exhibit increased autophagy in response to the stress stimuli induced by vincristine.

On the other hand, treatment with rapamycin, an autophagy inducer, decreased vincristine resistance in nonsilenced and shRNA2-silenced cells (Fig. 6E). This finding indicates that compensation for the impaired autophagy observed in shRNA2 cells may be a strategy to attenuate drug resistance in this cell type. However, this was not the case with doxorubicin (Fig. 6E).

When ACN cells were cultured under serum deprivation and in low glucose conditions, in comparison to high glucose plus serum supplementation, cell death rates in response to vincristine treatment increased independently of P2X7 receptor expression silencing (Fig. 6F). However, for doxorubicin, this shift in cell death upon starvation was observed only in shRNA2-silenced cells (Fig. 6F), suggesting that this attenuation in doxorubicin resistance may be related to starvation-induced autophagy upregulation.

Figure 6. Culture conditions and autophagy in drug resistance. A) Contributions of culture conditions and P2X7 receptor isoform silencing to the ACN cell drug response to doxorubicin (doxo) or vincristine (vinc) (n=3). B) Cell viability percentage ratio of HEK 293 cells grown in serum-containing medium over serum deprivation, representing the serum-induced growth of HEK 293 cells according to the presence of P2X7 receptor isoforms (n=3). C) Cytometric measurements of the autophagy markers LC3 (n=4) and Beclin (n=3). D) Representative cytometry dot plot of LC3+ cells in the scrambled or shRNA2 cell group. E) Cell viability measurements of ACN cells treated with vincristine (n=5) or doxorubicin (n=3) in the presence or absence of rapamycin. F) Cell viability measurements of ACN cells treated with vincristine or doxorubicin with or without serum starvation (n=3). Values were calculated as detailed in Methods section item 2.2.5. $*p<0.05$; $**p<0.01$; $***p<0.001$.

2.3.4. P2X7A has an efflux-preventing role, and P2X7B is associated with higher efflux activity, probably mediated by MRP-type ABC transporters

To investigate whether P2X7 receptor-mediated drug resistance is related to efflux pumps, an efflux activity assay was performed. shRNA2-silenced cells showed the highest efflux ability, followed by shRNA1-silenced cells (Fig. 7). This suggests that P2X7A has an efflux-preventing role.
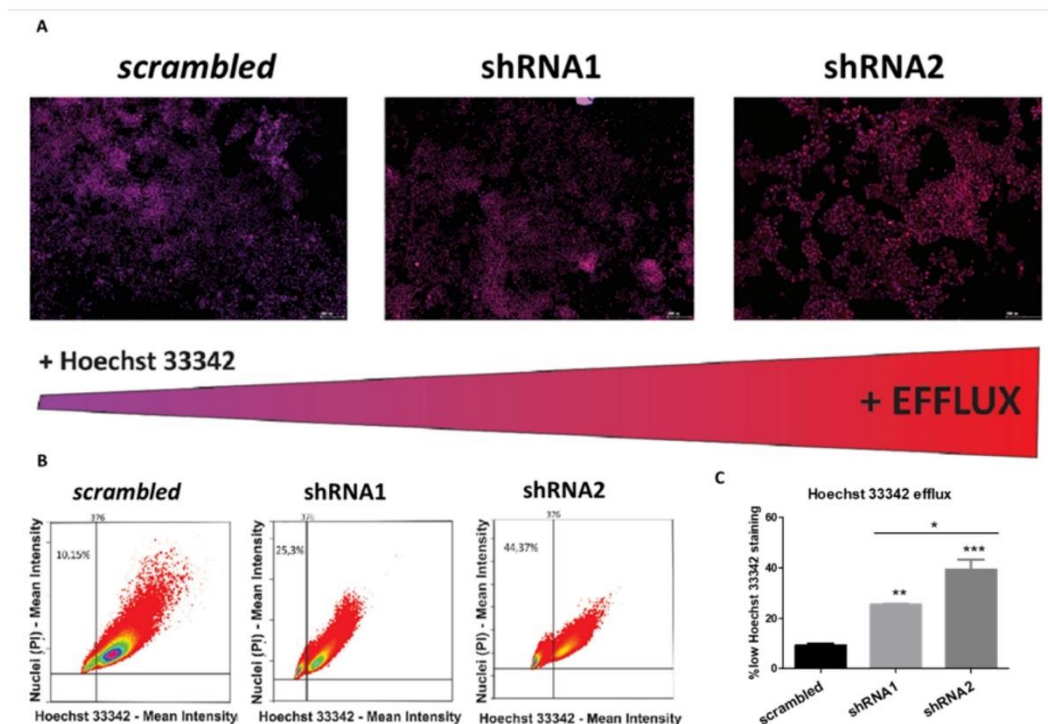
Figure 7. Effects of P2X7 receptor isoforms on the activity of drug efflux pumps in neuroblastoma cells. A) Fluorescence microscopy images of ACN cells differently silenced for P2X7 receptor isoforms costained with PI (nuclear marker, red) and Hoechst 33342 (efflux-prone dye, blue), obtained with a TissueFAXS® fluorescence microscope. Scale bars = 200 μM. The redness of cell nuclei is indicative of lower Hoechst 33342 staining and thus higher efflux activity. B) Dot plots quantifying the fluorescence intensity of PI and Hoechst 33342 on P2X7 receptor isoform-silenced or nonsilenced ACN cells. Thresholds set based on negative and positive control samples select a low Hoechst 33342-staining population. C) Percent representation of ACN cell populations with high efflux activity (low Hoechst 33342 staining). (n=3) *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Although P-gp is the most expressed efflux pump among those verified in this study, MRP pumps appear to be the most relevant for vincristine resistance in neuroblastoma cells, as all ACN cell types exhibited increased MRP1 expression upon vincristine treatment (Fig. 8A). In shRNA2-silenced cells, vincristine resistance was attenuated by MRP pump inhibition (probenecid) (Fig. 8B), and Bliss independence analysis showed that probenecid acts synergistically with vincristine, reducing drug resistance (Fig. 8C). P-gp inhibition only attenuated the drug resistance of shRNA1-silenced cells to doxorubicin, which does not seem to be a highly relevant resistance phenomenon because doxorubicin is highly effective in shRNA1-silenced cells (Fig. S3). When the BCRP antagonist was administered concomitantly with anticancer drugs, no relevant differences were observed.
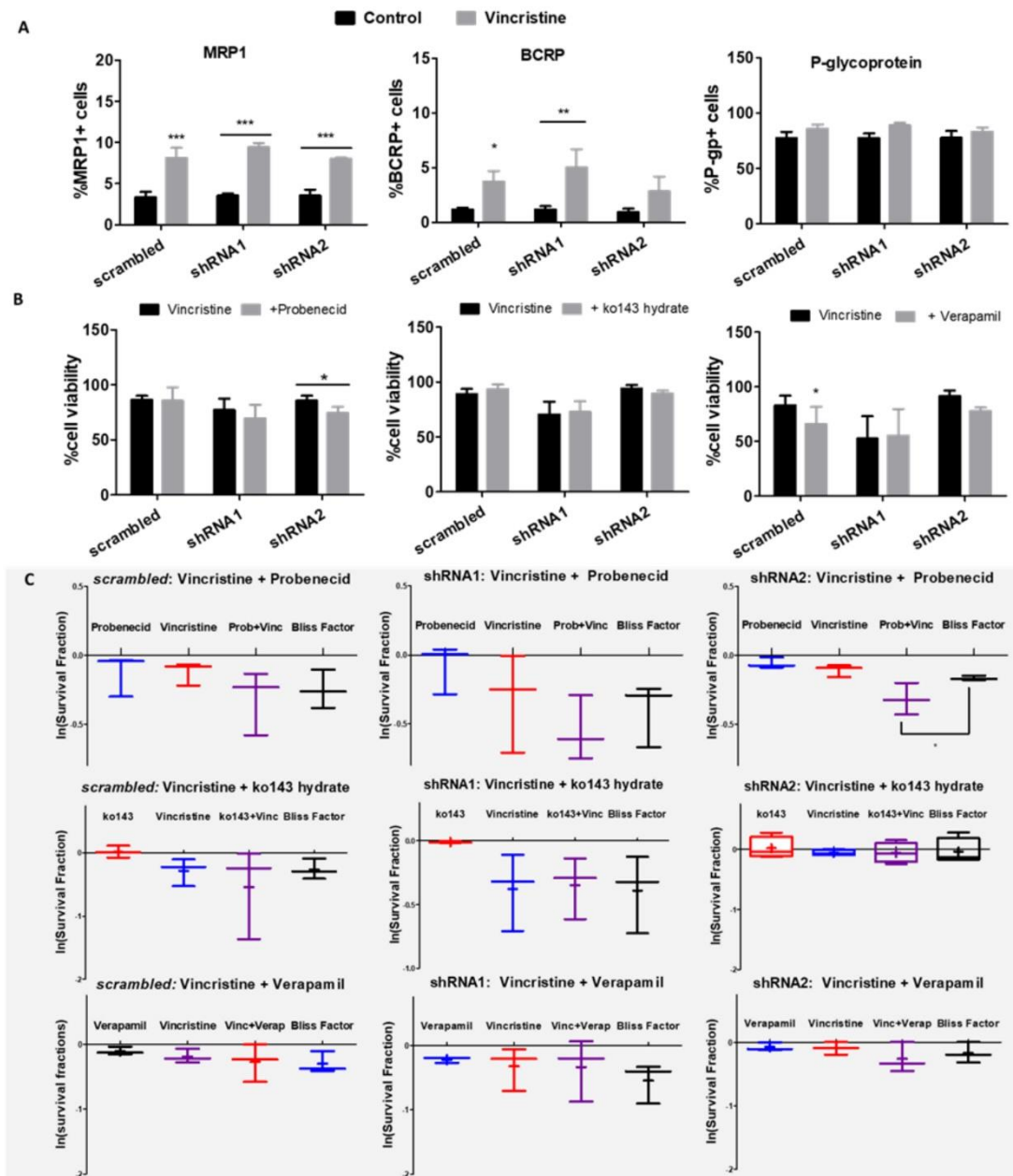
Figure 8. Functions of ABC transporters in neuroblastoma cells differentially expressing A and B isoforms. A) Cytometric measurements of MRP-1, BCRP and P-glycoprotein transporter-expressing ACN cells untreated vs. treated with vincristine (n≥3). B) Cell viability values of ACN cells treated with vincristine and/or the ABC transporter modulator probenecid (MRP-1), ko143-hydrate (BCRP) or verapamil (P-glycoprotein) (n≥3). C) Synergy investigation of vincristine combined with ABC transporter modulators in ACN cells using the Bliss Independence method (de Mello et al. 2022), based on the natural logarithm of experimental survival fractions of populations treated with both drugs in combination, compared to the predicted survival fraction called Bliss Factor (n≥3). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Upon EMT induction, HEK 293 *mock* cells raised MRP1 expression levels but decreased BCRP expression and was dependent on ATP treatment to raise P-gp levels, an obviously not P2X7 receptor-mediated effect. When the P2X7A isoform was overexpressed, MRP1 and BCRP levels responded to EMT induction similarly to *mock* cells. However, P-gp levels were reduced, possibly due to P2X7A overexpression. In the case of P2X7B overexpression, the increased MRP1 levels upon EMT induction were dependent on the presence of ATP (Fig. 9), suggesting a critical role for the P2X7B variant in inducing MRP1 efflux pump expression, similar to the observed Twist2 expression patterns.
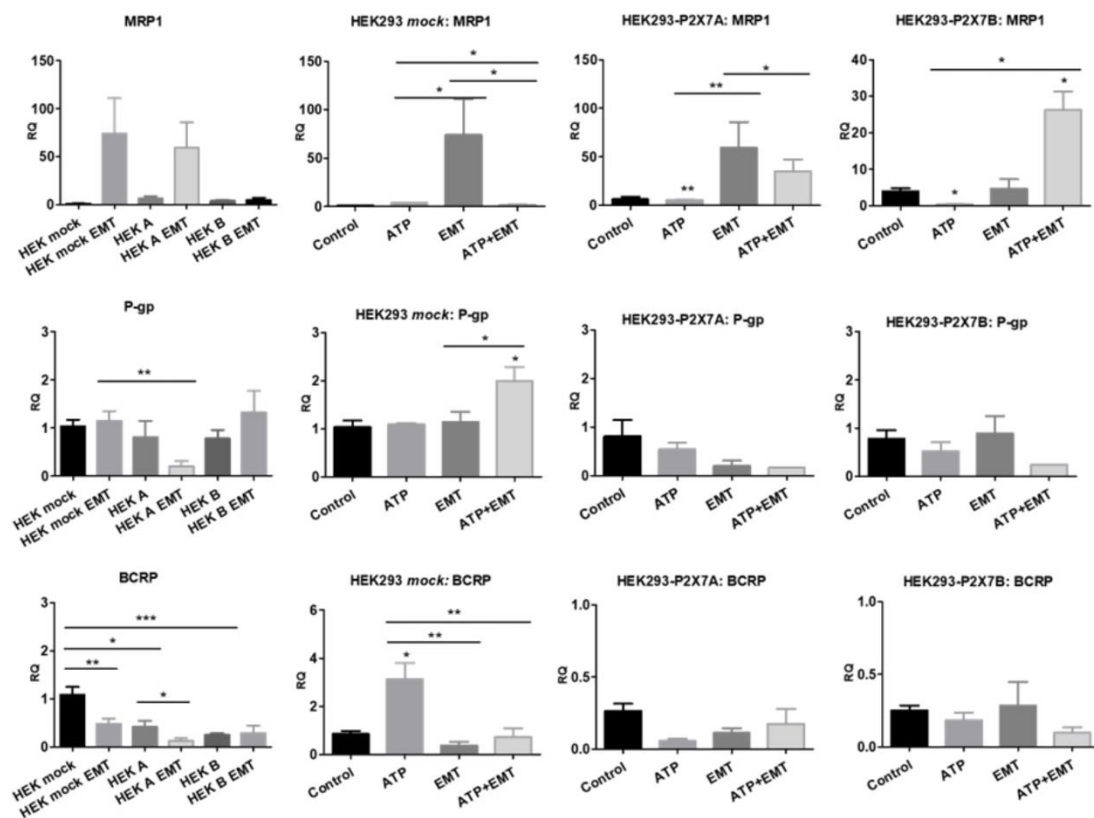
Figure 9. Contributions of P2X7 receptors and EMT to ABC transporter expression. mRNA expression levels of MRP1, P-glycoprotein (P-gp) and BCRP in HEK 293 cells treated or not treated with ATP and/or EMT inducers (EGF 50 ng/ml + TGF-β 5 ng/ml) (n≥3). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

2.4. Discussion

The therapeutic combination of vincristine, doxorubicin, and cyclophosphamide constitutes one of the cycles of induction therapy widely used for the treatment of high-risk neuroblastoma (National Cancer Institute - NIH). These drugs have complementary mechanisms of action, and the cytotoxicity of the combination is based mainly on targeting DNA (Škubník et al. 2021). When used individually, their effects are expected to diverge in terms of potency and specificity, considering the differences between their mechanisms and properties.

In our study, we observed high resistance of neuroblastoma cells to vincristine, which is a tubulin-binding compound that acts through microtubule disruption (Škubník et al. 2021). Vincristine is efficiently effluxed from cells via P-gp, leading to strong resistance to vincristine treatment in cell types overexpressing this efflux pump (Škubník et al. 2021). Indeed, we observed that >90% of the ACN neuroblastoma cell population expressed P-gp, regardless of P2X7 receptor isoform expression patterns.

The responses observed upon agonism or antagonism of the P2X7 receptor are consistent with previous findings. While the P2X7 receptor is widely known as a cell death-promoting receptor, absence of the complete C-terminal tail prevents macropore opening, compromising the ability to induce cell lysis (Adinolfi et al. 2005; Feng et al. 2006; Di Virgilio et al. 2018; Arnaud-Sampaio et al. 2020b). Whenever both isoforms A and B are expressed, they tend to coassemble, forming a heterotrimeric receptor. In this particular setting, the B isoform may enhance the responses mediated by P2X7A (Adinolfi et al. 2010). Additionally, P2X7B expression alone is the phenotype mostly related to growth promotion (Giuliani et al. 2014), supporting our evidence implicating P2X7B isoform importance in drug resistance. Consistently, we observed that P2X7A-

expressing HEK 293 cells have higher death ratios in response to chemotherapy drugs. ACN cells with basal expression of the P2X7 receptor were more drug resistant than their counterparts silenced for both isoforms A and B, also corroborating the survival and proliferation functions previously observed for endogenous expression of the P2X7 receptor (Baricordi et al. 1999; Di Virgilio et al. 2018). Recent studies have suggested a role for P2X7B in resistance to daunorubicin and radiotherapy, such that cancer treatments that increase the extracellular ATP levels in the TME may lead to death of P2X7A-overexpressing cells while promoting the survival of cells overexpressing P2X7B (Pegoraro et al. 2020; Zanoni et al. 2022).

Here, we demonstrated that P2X7A is crucial for shifting the cell phenotype toward a more differentiated state, reducing cell stemness in response to retinoic acid. This finding is complementary to what was observed by Glaser et al: P2X7 receptor expression in embryonic cells was suppressed to allow neural differentiation (Glaser et al. 2014). The most relevant isoform in this case is P2X7A, and when this isoform is absent in immature cells, differentiation is not triggered. Indeed, when used for neuroblastoma treatment, retinoic acid also faces resistance, a phenomenon termed retinoid resistance (Duffy et al. 2017). If P2X7A expression relates to retinoid acid sensitivity, the detection of this isoform in the tumor mass might be a marker for guiding treatment decisions. Combining retinoic acid treatment with a P2X7 receptor agonist could also be a promising approach and is worth further investigation. Further preclinical studies could explore whether P2X7 receptor agonism together with retinoic therapy, prior to chemotherapy, would modulate the tumor mass toward a more drug-responsive phenotype, avoiding the selection of less differentiated resistant cells. Although there are still no specific agonists or antagonists for each isoform, both of

them seem to be necessary for cell differentiation, meaning that a general agonist might work.

On the other hand, the P2X7B isoform was related to enhanced expression of EMT markers upon stimulation with well-known EMT inducers and increased EMT-triggered drug resistance. This finding is absolutely novel. Although relationships between EMT, invasiveness and the P2X7 receptor have been demonstrated (Ziberi et al. 2019; Arnaud-Sampaio et al. 2020b; Zhang et al. 2021), investigation of the involved isoforms has never been pursued. Because P2X7B is a main player in drug resistance and is also implicated in EMT, targeting this isoform in cancer seems promising for preventing EMT, thus overcoming drug resistance and tumor relapse.

shRNA2-silenced neuroblastoma cells expressing only the P2X7B isoform showed decreased levels of autophagy markers. Induction of autophagy either pharmacologically or through starvation decreased resistance to the drugs used in our study, suggesting that this autophagy impairment may be an actual contributor to neuroblastoma cell resistance. Although it is usually thought that autophagy would be cytoprotective and thus enhance resistance, autophagy triggering is a mechanism of inducing cell death. Indeed, enhanced sensitivity to doxorubicin when autophagy is inhibited has been reported for several cancers (Chen et al. 2018), and increased autophagy levels in response to a class I phosphatidylinositol 3 kinase/mTORC1 inhibitor enhanced doxorubicin-induced apoptosis of neuroblastoma cells (Westhoff et al. 2013), consistent with our observations. Regarding vincristine, inhibition of autophagy is also generally beneficial to decrease cancer cell drug resistance (Škubník et al. 2021).

Previous evidence points to the P2X7 receptor either as a positive or negative regulator of autophagy (Takenouchi et al. 2009; Sun et al. 2015; Young et al. 2015; Fabbrizio et al. 2017; Orioli et al. 2017; Arnaud-Sampaio et al. 2020b). In one study, P2X7 receptor agonism enhanced autophagic flux in a macropore-dependent way, leading to cell death, which was prevented by autophagy inhibition (Young et al. 2015). In other words, the activation tonus of the receptor was decisive: while autophagy was increased upon tonic short-term stimulation with ATP, sustained stimulation with higher concentrations decreased autophagic flux (Fabbrizio et al. 2017). Once more, adding the isoforms to the equation helps clarify these divergences: while the P2X7A receptor is crucial to macropore-dependent autophagy-mediated cell death, as Fabbrizio et al. have shown (Fabbrizio et al. 2017), P2X7B receptor-expressing cells have lower autophagy levels and thus are less susceptible to autophagy-mediated cell death (Fig. 10), as suggested by our data.

While the P2X7A receptor was important for preventing efflux, the P2X7B isoform was related to the highest efflux phenotype in neuroblastoma cells. This finding reinforces the perspective that for several functions, A and B isoforms have counterbalancing roles, as demonstrated for drug susceptibility, EMT, cell differentiation, and autophagy (Fig. 10). This difference in efflux activity is most likely mediated by MRP-type pumps, as probenecid increased the susceptibility of P2X7B-expressing cells to vincristine. It has been demonstrated that treatment with retinoic acid decreases MRP1 expression levels in neuroblastoma cells (Bordow Sharon B et al. 1994), which is consistent with our findings and helps conciliate all the evidence observed in the present work. If the absence of the P2X7A isoform maintains cells in a pluripotent state, as suggested by our data, and P2X7B isoform expression increases drug resistance partly through MRP-type pump modulation, the inability of retinoic acid treatment to decrease

pluripotency and thus MRP1 expression may be related to its inability to decrease drug resistance. In P2X7A variant-expressing cells, however, retinoic acid successfully decreased pluripotency and possibly MRP1 levels, as observed in the aforementioned study, explaining the reduced drug resistance.

Figure 10. Schematic representation of the roles of splice variants of the p2rx7 gene in neuroblastoma chemoresistance. The alternative splicing of the p2rx7 gene localized on chromosome 12 generates several mRNA variants that are translated into nonfunctional or only two well-characterized functional ion channels, the A and B isoforms. P2X7A corresponds to the full-length variant, and P2X7B is produced by the retention of an intron containing a stop codon, which shortens the protein length. This truncated version lacks the C-terminal tail that has been described as crucial to macropore opening observed in cells expressing P2X7A. These structural differences in splice variants determine distinct contributions of the P2X7 receptor to neuroblastoma drug resistance. The P2X7A receptor is crucial for macropore-dependent autophagy-mediated cell death and quiescence induction and downregulates efflux pumps. P2X7A favors retinoid sensitivity for neuroblastoma cell differentiation and epithelial

phenotype. The B isoform plays a primary role in cell proliferation 16 to favor drug resistance. The P2X7B receptor is important for resistance to retinoids, retaining cells in a stem-like phenotype, and efflux of drugs via MRP-type transporters. B isoform-expressing cells show impaired autophagy and a mesenchymal-prone phenotype. Taken together, these findings reveal counterbalancing roles for the A and B isoforms in neuroblastoma cells. Created with BioRender.com.

2.5. Conclusion

Tumor malignancy depends on a complex setting of characteristics shared by its constituent cells. Cancer cell stemness, mesenchymal-epithelial grade, efflux activity, metabolic regulation, and crosstalk with stromal cells are just a few countless processes that influence tumor aggressiveness. Following the conclusion that the P2X7 receptor isoform expression pattern was critical for drug resistance of neuroblastoma cells, we observed some of the main candidates that could explain P2X7B-mediated drug resistance. Our results point to cooperatively built drug resistance, which is the result of many aspects that individually present only moderate effects but together build up a relevantly resistant phenotype. Given the pleiotropic role of the P2X7 receptor in modulating various cellular functions, this is not surprising. On the one hand, we demonstrated that P2X7A participates in triggering neuroblastoma cell differentiation, retinoid sensitivity and autophagy and downregulates efflux, and on the other hand, we showed the complementary role of P2X7B in suppressing autophagy, inducing drug efflux, and promoting EMT (Fig. 10). The present work is thus highly relevant for resolving controversial findings in previous studies and proposing an integrated perspective to explore the P2X7 receptor.

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4. Attachment list

4.1. Supplementary material

4.2. Published journal articles:

4.2.1. P2X7 receptor isoform B is a key drug resistance mediator for neuroblastoma. Frontiers in Oncology, 2022. Arnaud-Sampaio, VF; Bento, CA; Glaser, T; Adinolfi, E; Ulrich, H; Lameu, C.

4.2.2. The P2X7 Receptor in the Maintenance of Cancer Stem Cells, Chemoresistance and Metastasis. Stem Cell Reviews and Reports, 2020. Arnaud-Sampaio, VF; Rabelo, ILA; Ulrich, H; Lameu, C.

4.2.3. Using cytometry for investigation of purinergic signaling in tumor-associated macrophages. Cytometry Part A, 2020. Arnaud-Sampaio, VF; Rabelo, ILA; Bento, CA; Glaser, T; Bezerra, J; Coutinho-Silva, R; Ulrich, H; Lameu, C.

4.2.4. Calcium signalling: A common target in neurological disorders and neurogenesis. Seminars in Cell & Developmental Biology, 2018. . Glaser, T; Arnaud-Sampaio, VF; Lameu, C; Ulrich, H.

4.2.5. P2X7 receptor: the central hub of brain diseases. Frontiers in Molecular Neuroscience, 2020. Andrejew, R; Oliveira-Giacomello, A; Ribeiro, D; Glaser, T; Arnaud-Sampaio, VF; Lameu, C; Ulrich, H.

4.2.6. ATP and spontaneous calcium oscillation control neural stem cell fate determination in Huntington's disease: a novel approach for cell clock research. Molecular Psychiatry, 2020. Ulrich, H; Glaser, T; Shimojo, H; Ribeiro, D; Martins, P;

Beco, R; Kosinski, M; Arnaud-Sampaio, VF; Correa-Veloso, J; Oliveira-Giacomelli, A; Lameu, C; Santos, AP; de Souza, H; Teng, Y; Kageyama, R.

4.2.7. Hyperactivation of P2X7 receptors as a culprit of COVID-19 neuropathology. Molecular Psychiatry, 2021. Ribeiro, DE; Oliveira-Giacomelli, A; Glaser, T; Arnaud-Sampaio, VF; Andrejew, R; Dieckmann, L; Baranova, J; Lameu, C; Ratajczal, M; Ulrich, H.

4.2.8. Cancer Metabostemness and Metabolic Reprogramming via P2X7 Receptor. Cells, 2021. Rabelo, ILA; Arnaud-Sampaio, VF; Adinolfi, E; Ulrich, H; Lameu, C.

4.2.9. Complex diseases demand novel treatment strategies: understanding drug combination. Drug Combination Therapy, 2022. De Mello, RS; Arnaud-Sampaio, VF; Maciel, LF; de Sá, V; Glaser, T; Ulrich, H; Lameu, C.

ATTACHMENTS

4.1. Supplementary material

A

B
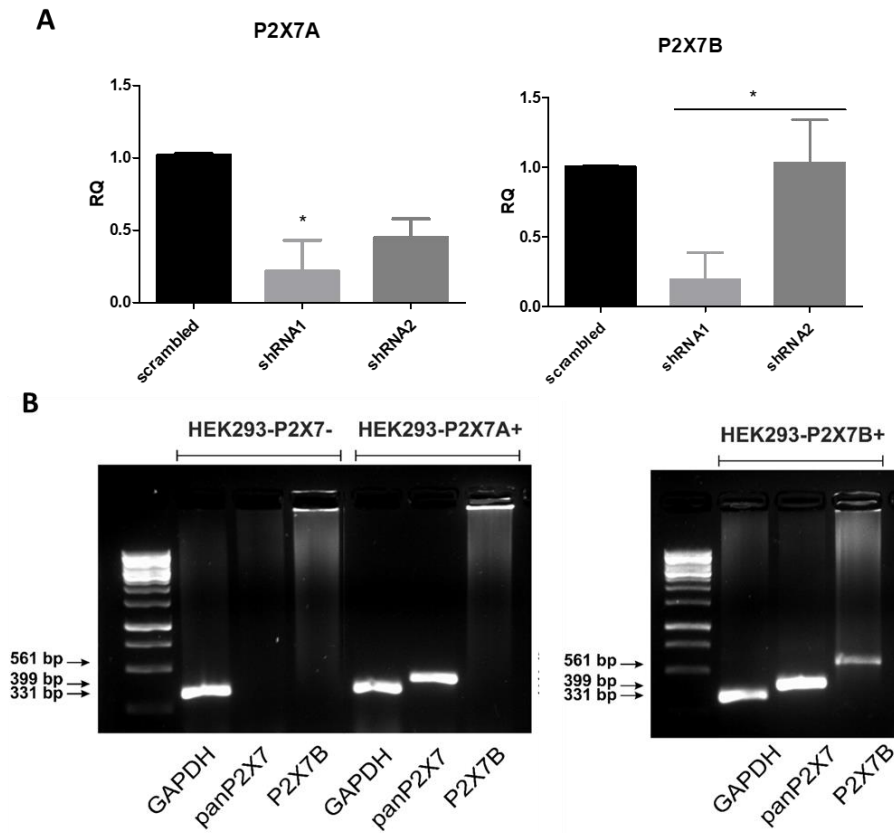
Figure S1. Differential expression of isoforms A and B in neuroblastoma cells and HEK 293 cells. A) mRNA expression levels of P2X7 receptor isoforms A and B in neuroblastoma cells stably transfected with the small hairpin RNAs scrambled, shRNA1 and shRNA2 (sequences described in Table 1); n=3. B) Agarose gel electrophoresis picture of PCR-reaction products performed for detection of P2X7 receptor isoforms in HEK 293 cells transfected for overexpression of isoforms A or B. *p<0.05 compare to scrambled.
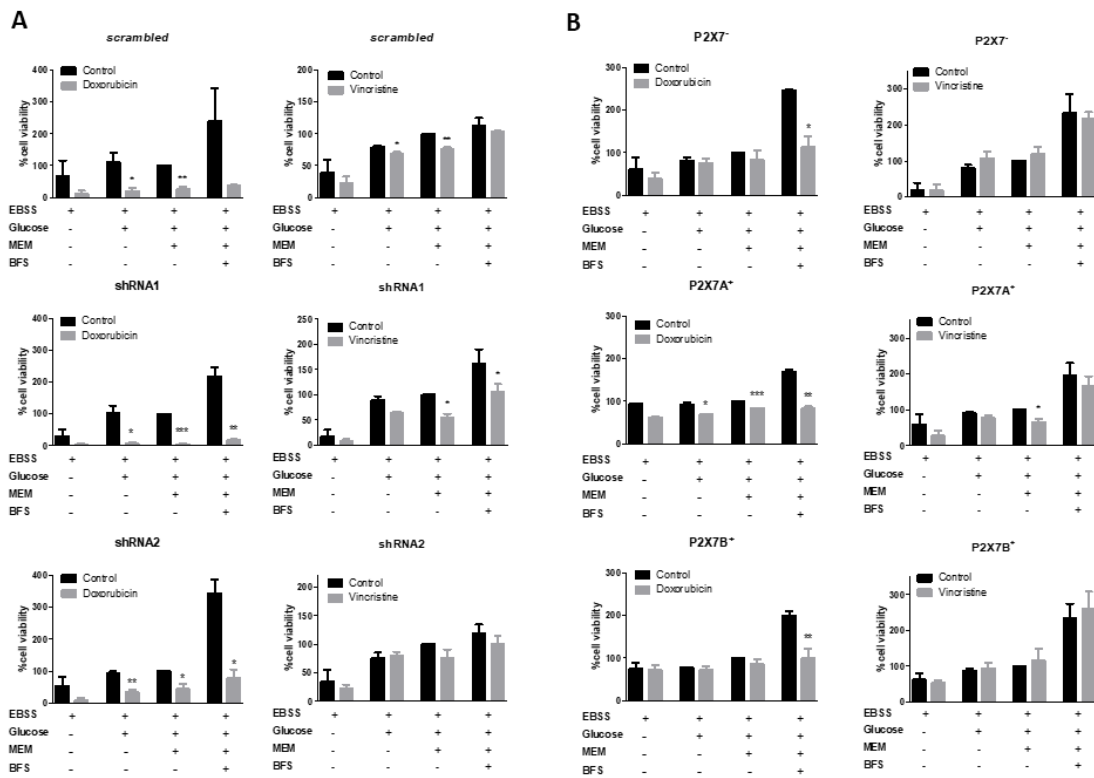
Figure S2. A) Cell viability values of ACN cells treated with vincristine or doxorubicin in progressively enriched culture conditions. MEM-EBSS medium was used as the control condition for normalization of cell viability values (n=3). B) Cell viability values of HEK293 cells treated with vincristine or doxorubicin in progressively enriched culture conditions. MEM-EBSS medium was used as the control condition for normalization of cell viability values (n=3). *p<0.05; **p<0.01; ***p<0.001.
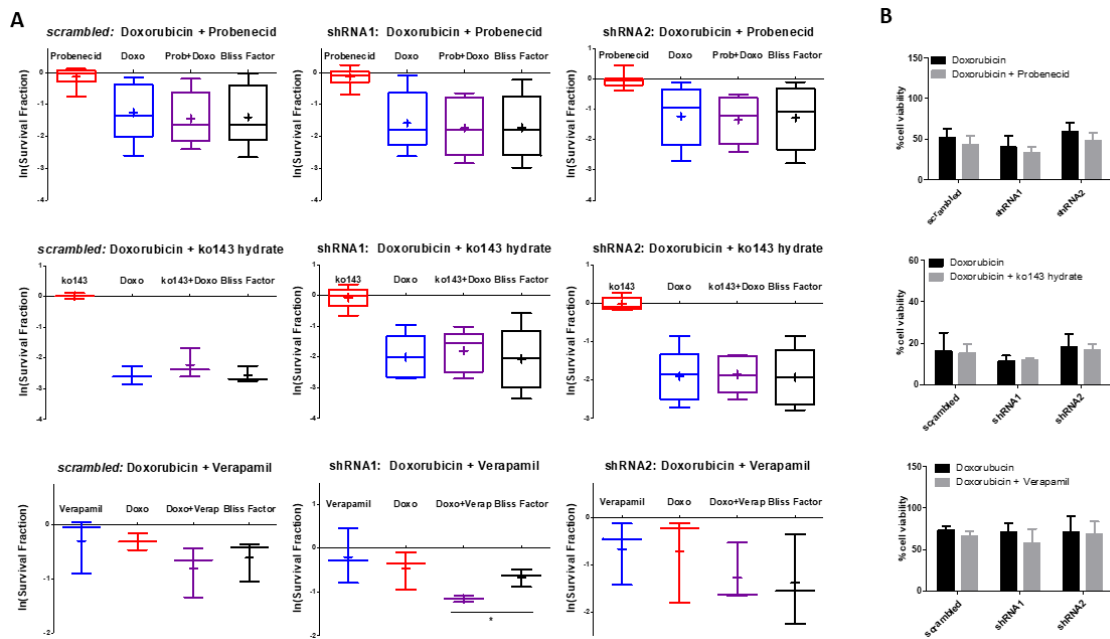
Figure S3. A) Synergy investigation of doxorubicin combined with ABC transporter modulators in ACN cells using the Bliss Independence method based on the natural logarithm of experimental survival fractions of populations treated with both drugs in combination compared to the predicted survival fraction called the Bliss Factor (n>=3). B) Cell viability values of ACN cells treated with vincristine and/or the ABC transporter modulators probenecid, ko143-hydrate or verapamil (n>=3). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

4.2. Published journal articles

4.2.1. P2X7 receptor isoform B is a key drug resistance mediator for neuroblastoma. *Frontiers in Oncology*, 2022.

Check for updates

P2X7 receptor isoform B is a key drug resistance mediator for neuroblastoma

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Drug resistance is a major challenge for all oncological treatments that involve the use of cytotoxic agents. Recent therapeutic alternatives cannot circumvent the ability of cancer cells to adapt or alter the natural selection of resistant cells, so the problem persists. In neuroblastoma, recurrence can occur in up to 50% of high-risk patients. Therefore, the identification of novel therapeutic targets capable of modulating survival or death following classical antitumor interventions is crucial to address this problem. In this study, we investigated the role of the P2X7 receptor in chemoresistance. Here, we elucidated the contributions of P2X7 receptor A and B isoforms to neuroblastoma chemoresistance, demonstrating that the B isoform favors resistance through a combination of mechanisms involving drug efflux *via* MRP-type transporters, resistance to retinoids, retaining cells in a stem-like phenotype, suppression of autophagy, and EMT induction, while the A isoform has opposite and complementary roles.

KEYWORDS

P2X7 receptor isoforms, chemoresistance, childhood cancer, cancer, drug efflux, cancer stem cells, autophagy, epithelial-mesenchymal transition

Introduction

Drug resistance is undoubtedly the greatest challenge in the fight against cancer (1). Although new treatment options are progressively developed, few surviving cancer cells are enough to promote disease relapse, and the multiple factors that influence the ability of cancer cells to evade therapy are highly complex. In the case of neuroblastoma (NB), a childhood tumor affecting cells of the sympathetic peripheral nervous system, nearly 50% of high-risk patients either relapse or do not respond to first-line therapy protocols (2). Therefore, the investigation of molecular targets involved in cancer cell fate

determination, which shift cells toward either death or survival, is crucial for advancing cancer research.

The P2X7 receptor, an ion channel of the P2X family of ATP-gated ionotropic purinergic receptors, is of particular interest given its long-known cell death-inducing properties (3). Thus, it has been previously hypothesized that P2X7 receptor stimulation in cancer cells could present a survival challenge, contributing to successful cancer therapy. Indeed, P2X7 receptor upregulation in brain tumor cells is a good prognostic predictor for radiation therapy response, and P2X7 receptor agonists have been shown to potentialize the cytotoxic effect of anticancer agents (4–6).

The cell death-promoting role of the P2X7 receptor depends on the formation of a large nonselective membrane pore that is permeable to molecules of up to 900 Da. Macropore opening is triggered by sustained receptor stimulation and leads both to altered membrane permeability, allowing the influx of large molecules, and to ATP efflux (7), increasing the ATP concentration in the extracellular space.

However, very high concentrations of extracellular ATP (5-10 mM) are required to activate the cytotoxic activity of the P2X7 receptor, and although these concentrations are probably achieved following classical anticancer therapies that lead to necrosis, such as chemotherapy or radiotherapy, they are rarely present in the untreated tumor microenvironment (TME) due to the activity of ubiquitous ectonucleotidases (3, 8). Therefore, the P2X7 receptor is usually active only as an ion channel within the TME, and as such, it has been shown to promote cell growth, neovascularization, matrix degradation and metastasis in various solid and liquid tumor models, including neuroblastoma (3, 9–11).

In addition to the biphasic gating of the P2X7 receptor ion channel, the existence of distinct splice variants of the *p2rx7* gene also plays a role in determining distinct cellular responses (3). Among the isoforms generated by alternative splicing of the human *p2rx7* gene, the A and B isoforms are the only well-characterized functional ion channels and are ubiquitously expressed (12). While P2X7A corresponds to the full-length variant, P2X7B arises from the retention of an intron containing a stop codon, which shortens the protein length (13, 14). This truncated version lacks the C-terminal tail that has been described as crucial to macropore opening.

However, the P2X7B variant is still functional, as it retains the capability of opening the ion channel, leading to downstream cellular effects (14, 15). In previous studies, the P2X7A variant has produced death-promoting antitumor effects, while the P2X7B variant has been implicated in the trophic, metastatic and resistance-prone properties of tumor cells (16–18). Furthermore, our group has observed that the malignancy of NB cells is related to increased P2X7B expression (19).

Therefore, we aimed to resolve the differential contributions of P2X7 receptor splice variants to the drug resistance of NB by further analyzing the mechanisms involved. Our results point to multifaceted drug resistance, comprising many mechanisms that together build up a consistent resistant phenotype. On the one hand, we demonstrated that P2X7A favors retinoid sensitivity to NB-cell differentiation and autophagy and downregulates efflux pumps. On the other hand, we showed the complementary role of the P2X7B isotype in suppressing autophagy, inducing drug efflux, and favoring EMT. The obtained results are highly relevant for explaining the dual role of the P2X7 receptor in tumor biology, paving the way for innovative therapies for chemoresistant tumors.

Materials and methods

1. Cell lines

ACN human neuroblastoma cells derived from bone marrow metastasis (RRID : CVCL_1068) and HEK 293 cells (ATCC® CRL-1573™) were genetically modified as previously described (16, 20). ACN cells were silenced with shRNAs from OriGene™ as detailed in Table 1, resulting in three derived cell lines.

HEK 293 cells, which do not natively express P2X receptors, were transfected for P2X7 receptor A or B isoform expression, resulting in the following derived cell lines: HEK 293-A, expressing P2X7A; HEK 293-B, expressing P2X7B; and HEK 293-*mock*, not expressing any P2X7 receptor isoform. Procedures for obtaining transfection constructs are described in detail elsewhere (16).

TABLE 1 Target sequences of shRNAs used for ACN cell silencing.

	Target sequence (5'-3')	Predicted phenotype
shRNA1 c.n. TI202483	ACGTTTGCTTTGCTCTGGTGAGTGACAAG	P2X7A⁻/B⁻
shRNA2 c.n. TI202486	CATTAGGATGGTGAACCAGCAGCTACTAG	P2X7A⁻/B⁺
scrambled **shRNA** c.n. TR30012	–	P2X7A⁺/B⁺

2. Cell culture

The culture medium for ACN cells consisted of RPMI 1460 medium supplemented with 10% bovine fetal serum, 1% NEAA 100X solution (Gibco™, c.n. 15140122), 100 U/mL penicillin, 100 mg/mL streptomycin, and the clone selection antibiotic puromycin at a 500 nM final concentration. Cells were kept at 37°C and 5% CO_2.

HEK 293 cells were cultivated in DMEM/F12 culture medium supplemented with 10% bovine fetal serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and specific clone selection antibiotics (G48 sulfate at 200 ng/ml for HEK 293-A and HEK 293-mock cells and hygromycin B at 100 µg/ml for HEK 293-B cells). Cells were kept at 37°C and 5% CO_2.

3. Cell line characterization

For cell line validation and characterization of P2X7 receptor isoform expression patterns, PCRs were performed.

For ACN cells, TaqMan™ real-time RT–PCR assays were conducted. After RNA extraction and cDNA synthesis, as described below in the specific section, Taqman™ reactions were prepared containing 1 µl of cDNA, 1X Taqman™ Fast Advanced Master Mix, Applied Biosystems™, 1.8 µM final concentration of primers, 0.5 µM of FAM-MGB probe, and 1X GAPDH predesigned Taqman™ assay Hs02758991_g1 VIC-MGB. Cycling conditions consisted of 2 min at 50°C, 2 min at 95°C, followed by 40 cycles of 1 s at 95°C plus 20 s at 60°C in the thermocycler StepOnePlus®, Life Technologies. For quantification, we used the calculation method $2^{-\Delta\Delta Ct}$, normalizing scrambled-transfected sample values to 1.

The probe sequences were as follows: P2X7A 5′ CACAGCGGCCAGACCG 3′ - 6FAM; P2X7B 5′ ACAAGCG CTGCGTTAGT 3′ - 6FAM.

The primers used were as follows: P2X7A Forward 5′ CGGCTCAACCCTCTCCTACT-3′; P2X7A Reverse 5′ GG AGTAAGTGTCGATGAGGAAGTC-3′; P2X7B Forward 5′GGAAAATGGTTTGGAGAAGGAAGTG-3′; and P2X7B Reverse 5′CGATGAGGAAGTCGATGAACACA-3′.

For HEK 293 cells, conventional reverse transcription PCRs were used. After RNA extraction and cDNA synthesis, as described below in the specific section, PCRs were prepared with 1.25 U Taq DNA polymerase, Invitrogen™ in 1X Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, forward and reverse primers at 0.5 µM each, and 1 µL of cDNA in a 25 µL reaction. After denaturation at 94°C for 2 min, the reaction proceeded with 40 cycles of 94°C for 1 min + 50°C for 1 min + 68°C for 2 min. Finally, a cycle of 68°C for 10 min was conducted to guarantee the final extension of DNA strands.

The obtained products were subjected to electrophoresis in a 1.5% agarose gel prepared with TAE buffer. Samples were run at 80 V for 30 min, and images were obtained on a UV transilluminator.

The primers used were as follows: P2X7B Forward: 5′CCCATCGAGGCAGTGGA 3′; P2X7B Reverse: 5′ TAAAGC ATGGAAAAGAGAATCTC 3′; panP2X7 Forward: 5′ AGATCG TGGAGAATGGAGTG 3′; panP2X7 Reverse: 5′ TTCTCGTG GTGTAGTTGTGG 3′; GAPDH Forward: 5′ CCTCTGACTT CAACAGCGAC 3′; GAPDH Reverse: 5′ CATGACAAGGT GCGGCTCCC 3′.

4. Tumorsphere culture

For culturing cells as tumorspheres, cells were seeded in 0.2% F-127 pluronic acid-coated hydrophobic suspension dishes in defined DMEM High medium containing 20 ng/ml EGF, 20 ng/ml FGF and 1X N-2 supplement, Gibco™, for at least 96 h. For flow cytometry, tumorspheres were dissociated with 2 mM EDTA and vigorously pipetted.

5. Cell viability – alamarBlue®

For cell viability assessments, cells were seeded on 96-well plates with black borders and clear bottoms at a density of 10^4 cells per well with complete medium overnight. After adherence and 2 h of starvation in serum-depleted medium, the chemotherapy drugs vincristine (300 nM) or doxorubicin (100 µM) were added, combined or not with treatments (described in Table 2 and detailed in each figure), in supplemented medium without clone selection antibiotics (unless specifically stated) for 48 h.

For comparison between culture conditions, cells were also subjected to 2 h of starvation in serum-depleted medium and then incubated with the tested culture medium. To observe the effects of nutrient availability on drug resistance, a culture condition gradient composed of four conditions was tested: EBSS buffer, EBSS buffer + 2 g/dL glucose, MEM-EBSS medium, and MEM-EBSS + 10% FBS. For autophagy induction through serum starvation, groups consisted of a control group cultured in RPMI 1640 10% FBS and a starved group cultured in RPMI 1640 without FBS.

After specific interventions, the cells were incubated for a 2 h period with a 1:10 alamarBlue® solution prepared in culture medium protected from light at 37°C and 5% CO_2. Fluorescence readings were performed in FlexStation III, Molecular Devices™, at 530-560/590 nm excitation/emission wavelengths, as specified by the manufacturer. Cell viability values were compared to the control group, considered as 100%.

When analyzing the influences of a specific drug (detailed in Table 2) or culture condition on the effects of vincristine or doxorubicin, calculations were performed as described. First, the

TABLE 2 Details of pharmacologic treatments other than chemotherapy drugs.

	Description	Concentration
ATP	P2X7 receptor agonist	1 mM
BzATP	P2X7 receptor agonist	100 μM
γ-S-ATP	P2X7 receptor agonist	100 μM
A438079	P2X7 receptor antagonist	10 μM
BBG	P2X7 receptor antagonist	300 nM
Retinoic Acid	Neural differentiation inducer	5 μM
TGF-β	EMT inducer	5 ng/ml
EGF	EMT inducer	50 ng/ml
Probenecid	MRP-type inhibitor	1 mM
Verapamil	Pgp inhibitor	5 μM
Ko143 hydrate	BCRP inhibitor	30 nM
Rapamycin	Autophagy inducer	200 nM

cell viability of the group treated with the additional drug alone was calculated considering the cell viability of the untreated group of the same cell line as 100%. A one-tailed paired t test analysis determined whether the treatment alone was cytotoxic considering a confidence interval of 95%.

If $p > 0.05$: Cell viability of all groups in the analysis was calculated considering the cell viability of the untreated group as 100% and compared altogether using one- or two-way ANOVA variance analysis, according to experimental design.

If $p < 0.05$, additional treatment was considered cytotoxic, and as such, groups that received the cytotoxic additional drug were normalized considering the cell viability of the group treated exclusively with the cytotoxic drug as 100%, and related groups were compared for statistically relevant differences using t tests (when only two samples) or ANOVA, according to the experimental design.

6. Cell death – propidium iodide (PI)

For cell death analysis, cells were seeded in 12-well plates at a density of 3×10^5 cells per well with complete medium and incubated overnight. After adherence and 2 h of starvation in serum-depleted medium, chemotherapy drugs were added, combined or not with the treatments detailed in Table 2, in supplemented medium without clone selection antibiotics (unless specifically stated) for 48 h. After detachment with 2 mM EDTA, cells were washed in PBS, passed through a 40 μm cell strainer, and stained for 10 min with a 2 μg/ml PI solution in PBS at 4°C. The positive control consisted of a mixed cell sample incubated in 2 μg/ml PI + 0.1% Triton X-100 PBS solution, while the negative control was a mixed cell sample incubated in pure PBS.

Cell death rates were determined in an Attune[TM] Acoustic Focusing Cytometer, Life Technologies[TM], with minimal acquisition of 50,000 events per sample at 200 μL/min flow speed. The obtained data were analyzed in FlowJo software (BD Biosciences).

7. Dose–response curves

To characterize the response pattern of the employed cell lines to the chemotherapy drugs, cell viability drug-response curves were performed employing crescent drug concentrations in intervals of 0.5 log units: 0-100 μM for vincristine, 0-10 mM for doxorubicin, and 0-30 mM for cyclophosphamide. For cell death (PI staining) drug-response curves, the concentration ranges were 0-10 μM for vincristine, 0-100 μM for doxorubicin, and 0-10 mM for cyclophosphamide. IC50 and EC50 concentrations were calculated based on nonlinear regression analysis performed in GraphPad Prism 5[TM] software.

8. RNA extraction

For relative gene expression analysis, cells were seeded on 6-well plates at a density of 3×10^6 cells per well. After adherence and 2 h of starvation in serum-depleted medium, the respective treatments were applied in supplemented medium without clone selection antibiotics (unless specifically stated).

The cells were then washed with PBS, collected directly in TRIzol and frozen at -80°C until extraction with cold chloroform, precipitated in isopropanol and washed in 75% ethanol, as instructed by the manufacturer's protocol.

9. cDNA synthesis and relative expression analysis – RT–qPCR

Reverse transcription reactions using both oligoDT and random hexamers were performed after treating RNA samples

with DNAse. We used a RevertAid® reverse Transcription Kit from Invitrogen (Thermo Fisher) to synthesize cDNA from 2 µg of purified RNA from each sample following the manufacturer's protocol. Cycling conditions consisted of 5 min at 65°C, followed by 10 min at 25°C, 42°C for 60 min and 70°C for 10 min.

Primer pairs with the sequences specified in Table 3 and SYBR Green Master Mix 2X reagent were incubated with 1 µl of 10X diluted cDNA synthesis product. The cycling conditions for amplification were 95°C for 1 min for denaturation, 40X 95°C for 30 s, and 60°C for 1 min, followed by melting curve analysis performed in 3°C increments. The thermocycler StepOnePlus®, Life Technologies, was used. For quantification, we used the calculation method $2^{-\Delta\Delta Ct}$, normalizing control sample values to 1. As an endogenous control, primers targeting the EMC7 gene were employed.

10. Protein expression analysis – flow cytometry

Protein expression levels were quantified by flow cytometry. Cells were detached from culture plates with 2 mM EDTA, fixed in PFA 4% for a minimum of 1 h, and blocked/permeabilized in 4% FBS + 0.1% Triton X-100 PBS solution. An overnight incubation was then performed with primary antibodies (Abcam© ab129450, ab3380, ab32574, ab207612, and ab1316; Molecular Probes® L10382) at a 1:100 dilution in 1% FBS PBS 1X solution, followed by staining with Alexa Fluor® 555 anti-mouse or Cy5® anti-

rabbit secondary antibodies in 4% FBS + 0,1% Triton X-100 PBS 1X solution for 15 min.

Flow cytometry measurements were performed in an Attune™ Acoustic Focusing Cytometer, Life Technologies™, with BL-2 or RL-1 filter settings (Alexa Fluor® 555 anti-mouse or Cy5® anti-rabbit, respectively). Fluorescence thresholds were determined by negative samples incubated with secondary antibody in FlowJo software (BD Biosciences).

11. Cell cycle analysis

Cells were detached from culture plates with 2 mM EDTA, washed in PBS, fixed in 70% ethanol for 1 h at 4°C, passed through a 40 µm cell strainer, blocked and permeabilized in 1% FBS + 0.1% Triton X-100 PBS 1X solution for 30 min with agitation. Next, the cells were incubated with anti-ki67 Millipore® antibody (AB9260) in 1% BFS + 0,1% Triton X-100 PBS 1X solution for 45 min with agitation and further incubated with Alexa Fluor® 555 anti-rabbit IgG antibody for 15 min. Finally, the cells were washed and incubated with RNAse A 50 µg/ml solution in 0.1% Triton X-100 + 0.5% Tween 20 PBS 1X solution for 15 min. Populations were measured in an Attune™ Acoustic Focusing Cytometer, Life Technologies™, with minimal acquisition of 50,000 events per sample at 200 µL/min. The obtained data were analyzed using the FlowJo software (BD Biosciences).

12. Efflux activity – Hoechst 33342® staining followed by image cytometry (TissueFAXS®)

For efflux activity assessments, cells were seeded on 96-well plates with black borders and clear bottoms at a density of 10^4 cells per well with complete medium overnight.

Efflux activity by ATP-binding cassette (ABC) transporters was measured using the dye Hoechst 33342 at a 100 ng/ml solution prepared in culture medium, followed by incubation for 30 min and fixation in 4% PFA. A propidium iodide solution (PI) at 3 µg/mL was used to stain cell nuclei. Plates were scanned in the TissueFAXS®, TissueGnostics, fluorescence microscope, and cytometric quantifications were performed by StrataQuest® software.

Detection of PI staining by the nuclei mask tool in StrataQuest® software allowed mapping of the cell nuclei area in the images. Next, the fluorescence intensity for Hoechst 33342 was measured in the determined area and visualized as dot plots. As a negative control, samples stained with PI only were employed, while a positive control was prepared by cell fixation with 4% PFA before Hoechst 33342 staining. Thus, the method identified low Hoechst 33342-stained populations that were able to expel the dye.

TABLE 3 qPCR primer sequences.

Target	Sequence (5'→ 3')
P-gp (ABCB1) Forward	CGTGGGGCAAGTCAGTTCA
P-gp(ABCB1) Reverse	TCCTTCCAATGTGTTCGGCA
MRP1 (ABCC1) Forward	ACTAGGAAGCAGCCGGTGAA
MRP1 (ABCC1) Reverse	CTTCTGTGGGGACTTGACGA
BCRP (ABCG2) Forward	TGAAAAGGATGTCTAAGCAGGGA
BCRP (ABCG2) Reverse	GCAGGCCCGTGGAACATAA
N-cadherin Forward	GCCCAAGACAAAGAGACCCA
N-cadherin Reverse	TCAACTTCTGCTGACTCCTTCA
Fibronectin Forward	TGGGCAACTCTGTCAACGAA
Fibronectin Reverse	CCACTCATCTCCAACGGCAT
Nanog Forward	AGAAAGAGGTCTCGTATTTGCTG
Nanog Reverse	ACACTCGGTGAAATCAGGGT
Twist2 Forward	GACAGCAGTGACATCGGACA
Twist2 Reverse	GACCCAGAAGAAAAATCCAAACAGA
Twist1 Forward	CCACTGAAAGGAAAGGCATCAC
Twist1 Reverse	TATGGTTTTGCAGGCCAGTT

13. Drug interaction analysis

For drug interaction analysis, we employed the Bliss Independence model followed by *t* tests comparing transformed values of predicted and observed survival fractions to determine the statistical relevance of the observations, as described elsewhere (21).

14. Statistical analysis

For the cell viability and efflux assays, variances among groups were compared by paired *t* tests and one- or two-way ANOVA according to the experimental design followed by Bonferroni post tests, considering a 95% confidence interval. For relative expression analysis, one-way ANOVA was employed, followed by Bonferroni post test, also considering the 95% confidence interval.

Results

The P2X7B isoform is implicated in drug resistance, while the P2X7A variant is related to cell death induction

To investigate the influence of the P2X7 receptor and its isoforms on drug resistance and to confirm the function of each isoform, we chose two cell lines: a human neuroblastoma (NB) cell line (ACN cells) and HEK 293 cells. When submitted to 48 h treatments with drugs commonly used for NB treatment, ACN cells responded well to doxorubicin, i.e., presented a significant decrease in their viability upon treatment, as expected in chemotherapy, but not to cyclophosphamide – which is also expected as cyclophosphamide is a prodrug activated by hepatic metabolism -and had pronounced resistance to vincristine, reaching a plateau with a surviving ~50% population observed in both cell viability and cell death assays (Figures 1A, B). Similarly, HEK 293 cells did not show significant death rates upon vincristine or cyclophosphamide treatment (Figure 1C).

A shRNA-based silencing approach was used to selectively decrease the expression levels of P2X7 receptor isoforms. shRNA1, with a sequence designed to silence P2X7 receptor A and B isoforms, successfully decreased their expression levels, whereas shRNA2 selectively silenced B isoform, also according to what was expected based on its sequence (Figure S1A). HEK 293 cells, a cell type endogenously lacking P2X7 receptors (22), were transfected with the coding sequence of the isoform of interest, as described in the Methods section, generating three cell line subtypes: *mock* cells (control), HEK 293-A cells (P2X7A

overexpression), and HEK 293-B cells (P2X7B overexpression) (Figure S1B).

In our experiments, shRNA1-silenced ACN cells were the most susceptible to both vincristine and doxorubicin treatments, presenting the highest decrease in cell viability (reminiscent viable population of 43.87% ± SE 5.2 (shRNA1) vs. 76.08% ± SE 6.75 (*scrambled*), and 69.08% ± SE 7.65 (shRNA2), *p*=0.0034) (Figure 1D). In contrast, shRNA2-silenced cells had the highest doxorubicin resistance (reminiscent viable population of 52.75 ± SE 11.37 (shRNA2) vs. 9.81 ± SE 2.76 (shRNA1), and 27.25 ± SE 4.72 (*scrambled*), *p*=0.0007), robustly demonstrated in several different culture conditions (Figures 1D and S2A).

For HEK 293 cells, isoform expression was also relevant to vincristine, but not to doxorubicin response. P2X7A overexpression was related to the highest decrease in cell viability upon vincristine treatment, reinforcing the previously demonstrated cell death-promoting roles of this isoform (Figure S2B).

Although these findings may sound controversial, they are indeed complementary. In the ACN NB-cell model, nonsilenced cells express the P2X7A isoform, but not exclusively: they also express P2X7B, and the trimeric receptor may occur in a heterogeneous composition, representing a scenario closer to reality. In the HEK 293 model, the isoforms occur alone and are overexpressed, isolating and highlighting the function of each isoform.

As P2X7 receptor activity in NB cells is less evident than that observed in the HEK 293 cell overexpression model, we treated ACN cells with P2X7 receptor agonists and antagonists. Vincristine treatment alone significantly decreased the viability of shRNA1 cells only (69.95% ± SE 7.99 vs. 100% in the control group, $p \leq 0.05$). However, the combination of vincristine and ATP, the endogenous P2X7 receptor agonist, promoted a similar effect in the nonsilenced cells (73.2% ± SE 9.46 vs. 60.73% ± SE 13.72 in the presence of ATP) (Figure 2A). This finding corroborates the role of the P2X7A isoform in promoting vincristine-induced death, as observed in HEK 293 cells overexpressing this isoform. However, ACN shRNA2 cells did not respond to vincristine either in presence or absence of ATP, suggesting that the P2X7B receptor alone is related to a drug-resistant phenotype (Figure 2A).

Increased doxorubicin resistance of nonsilenced or shRNA2-silenced cells was attenuated by antagonism with Brilliant Blue-G (BBG), while shRNA1-silenced cell drug sensitivity remained unchanged (Figure 2B). Regarding vincristine resistance, shRNA2-silenced cells showed decreased resistance upon selective antagonism with A438079, an effect that was not observed in nonsilenced cells, indicating that in this context drug resistance is related to the specifically assembled P2X7 receptor with the B isoform (Figure 2C).

FIGURE 1

Dose–response curve of chemotherapy in cells differentially expressing the P2X7 receptor and its isoforms. Dose–response curves of vincristine (n=6), doxorubicin (n=3) and cyclophosphamide (n=3) in neuroblastoma cells. **(A)** Viability assays and **(B)** cell death assays performed in ACN cells containing scrambled shRNA. **(C)** Drug-response curves of the chemotherapy drugs vincristine, doxorubicin and cyclophosphamide in HEK 293 cells obtained by PI staining quantification by flow cytometry. **(D)** Cell viability measurements of ACN cells expressing (scrambled), silenced for both A and isoforms (shRNA1) and silenced for B isoform (shRNA2) after treatment with vincristine or doxorubicin. Data normalized by the respective cell type untreated control (n>=3). *p<0.05; **p<0.01; ***p<0.001.

While the P2X7A isoform is critical for retinoid-induced neural differentiation of NB cells, the P2X7B variant is related to EMT marker upregulation in HEK 293 cells and enhanced EMT-induced drug resistance of NB cells

Because stemness and epithelial-mesenchymal transition (EMT) are closely related to chemoresistance (14), we characterized our cell lines, which differentially expressed P2X7 receptor isoforms, regarding the degree of differentiation and epithelial-mesenchymal phenotype transformation (Figure 3). We assessed the mRNA transcript levels of vimentin and fibronectin, which are mesenchymal phenotype markers, and E-cadherin protein levels, which are indicative of the epithelial-like phenotype (23). The mRNA levels of NANOG, a marker of neural progenitor cells (24), were used as an indicator of stemness for NB cells.

In ACN shRNA1-silenced cells, absence of the P2X7 receptor was related to increased expression of vimentin, fibronectin, and E-cadherin and decreased expression of NANOG (Figure 3A), pointing to a mixed EMT-MET

FIGURE 2

P2X7 receptor activity in response to chemotherapy. **(A)** Cell viability measurements of ACN cells stimulated or not with ATP, the endogenous P2X7 receptor agonist, alone or in combination with vincristine (n>=3). **(B)** Cell viability measurements of ACN cells stimulated with doxorubicin (doxo) in the presence or absence of the P2X7 receptor antagonist Brilliant Blue-G (BBG) (n>=4). **(C)** Cell viability measurements of ACN cells stimulated with vincristine in the presence or absence of the P2X7 receptor-specific antagonist A438079 (n>=4). Values were calculated as detailed in Methods section item 5. *$p<0.05$ and **$p<0.01$.

(mesenchymal-epithelial transition) phenotype that cannot be restricted to a simple polarizing definition. The presence of P2X7B alone was related to decreased NANOG and fibronectin levels compared to nonsilenced cells, suggesting that these cells were not intrinsically more mesenchymal- or stem-like than those expressing both isoforms (Figure 3A).

Treatment of ACN cells with BzATP, a stable P2X7 receptor agonist, increased E-cadherin levels in control cells, an effect that was not observed in either shRNA1- or shRNA2-silenced cells, suggesting an epithelial-prone P2X7A-related effect (Figure 3B).

When treated with EGF and TGF-β, two known EMT-inducing factors, we observed that the lowest concentrations tested (50 ng/ml and 5 ng/ml, respectively) were optimal for inducing EMT in our model, producing the expected increase in vimentin expression levels. For this reason, we used these concentrations henceforth (Figure 3C).

This increase in vimentin expression upon treatment with EMT-inducing growth factors matched the enrichment of P2X7

receptor expression (Figure 3C) and increased cell quiescence (G0 state) in nonsilenced cells (Figure 3D), culminating in decreased cell death with vincristine treatment (Figure 3E).

Importantly, EMT made NB cells more resistant to death induced by treatment with vincristine alone or in combination with BzATP, the P2X7 agonist. This finding suggests that the mesenchymal phenotype overcomes P2X7A receptor activation, which produces death-promoting antitumor effects that were observed in NB cells expressing P2X7A and not treated with EMT-inducing growth factors (Figure 2A).

HEK 293-A cells shifted the expression levels of Twist-2, a transcription factor indicative of EMT. This effect was prevented by ATP treatment, which was also observed in *mock* cells, demonstrating that this ATP-induced effect was not related to the P2X7 receptor. When HEK 293-B cells were induced to undergo EMT, N-cadherin levels increased, but Twist2 levels increased only with concomitant addition of ATP, suggesting that P2X7B favors EMT and that direct

FIGURE 3

P2X7 receptor isoforms in epithelial-mesenchymal transition and stemness of neuroblastoma cells. **(A)** mRNA expression levels of vimentin, fibronectin and NANOG (n=3) and protein levels of E-cadherin (n=3) measured in scrambled, shRNA1 and shRNA2 cells. **(B)** E-cadherin protein levels measured by flow cytometry in BzATP-treated vs. nontreated ACN cells (n=3). **(C)** Vimentin and P2X7 receptor mRNA expression levels in scrambled ACN cells treated with combinations of low/high concentrations of TGF-β (5 or 25 ng/ml) and EGF (50 or 100 ng/ml) (n=3). **(D)** Cell cycle phase distribution of scrambled ACN cells treated with EMT inducers at optimal concentrations (TGF-β 5 ng/ml + EGF 50 ng/ml) (n=3). **(E)** Cell death measurements (PI-staining) of scrambled ACN cells treated with the P2X7 receptor agonist BzATP and/or vincristine, with or without treatment with EMT inducers at optimal concentrations (TGF-β 5 ng/ml + EGF 50 ng/ml) (n=3). *p<0.05; **p<0.01; ***p<0.001.

stimulation with ATP enhances this effect, pushing cells toward a more mesenchymal phenotype. Because EMT did not occur in *mock* cells but only in P2X7B-overexpressing cells, we hypothesize that the acquisition of a mesenchymal phenotype is mediated by the B isoform, highlighting P2X7B as an EMT-favoring isoform (Figure 4A).

When ACN NB cells were EMT-induced and treated with either vincristine or doxorubicin, shRNA2 cells showed a significant increase in remaining cell viability (83.60% ± SE 8.79 vs. 70.95% ± SE 7.27 in nontreated cells, p=0.0443), an effect not observed in shRNA1 cells (Figure 4B), suggesting that cells predominantly expressing the P2X7B isoform undergo EMT more effectively and thus become more drug-resistant.

When the expression of VEGF-A receptor, an EMT marker, was assessed, we found no differences among the untreated cell types, but this receptor was enriched in all groups when submitted to vincristine treatment, reinforcing the relevance of EMT in vincristine resistance of NB cells (Figure 4C).

Cancer cells cultured as tumorspheres are often used as a model for cancer stem cell enrichment in culture. Therefore, we compared NB cells cultured in monolayers or as tumorspheres.

While shRNA1-silenced ACN cells cultured in monolayers responded better to vincristine when their counterparts were cultured as tumorspheres, isoform expression patterns were not relevant for drug responses, maintaining cell death rates within the same range for all cell types (Figure 4D). This means that whatever advantages P2X7-silenced cells had for treatment sensitivity, they were suppressed in tumorspheres, and these cells become as resistant as their nonsilenced or shRNA2-silenced counterparts. Therefore, phenotype modulation promoted by tumorsphere culture compensated for the absence of the P2X7 receptor. Thus, nonsilenced and shRNA2-silenced cells did not further increase drug resistance in face of these stimuli.

shRNA1-silenced cells, when cultured as tumorspheres, reduced vimentin expression levels to 33.8% of the levels observed in the monolayer culture (RQ of 1.42 ± SE 0.2

FIGURE 4

Epithelial-mesenchymal transition and stemness of cells cultured in monolayers or as tumorspheres. **(A)** mRNA expression levels of EMT-related transcription factors in HEK 293 cells, untreated, or treated with ATP and/or EMT inducers (EGF + TGF-β) (n=3). **(B)** Cell viability measurements of vincristine- (n>=5) or doxorubicin-treated (n=4) ACN cells in the presence or absence of EMT inducers. Values were calculated as detailed in Methods section item 5. **(C)** Expression levels of the VEGF-A receptor protein in ACN cells treated with or without vincristine. Measurements were performed by flow cytometry. (n=3) **(D)** Cell death ratio of ACN cells cultured in monolayers or as tumorspheres upon treatment with vincristine. For normalization of cell death intrinsic to tumorsphere manipulation, values were calculated as detailed in Methods section item 5. **(E)** mRNA expression levels of vimentin and fibronectin in ACN cells cultured in monolayers or as tumorspheres (n=3). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

(monolayer) vs. 0.48 ± SE 0.09 (tumorspheres), p=0.0021) (Figure 4E), which may be interpreted as an indication of lower EMT grade. This may appear controversial to the fact that these cells respond poorly to vincristine in comparison to monolayer-cultured cells. In addition, both nonsilenced and shRNA1 cells had reduced fibronectin levels when cultured as tumorspheres (Figure 4E). However, in our experiments, epithelial phenotype did not seem to be a good predictor of drug response, and EMT induction was only effective in enhancing drug resistance in shRNA2 cells, linking EMT-induced drug resistance to the P2X7B isoform.

In order to investigate NB-cell differentiation ability, considering that the ACN cell line is poorly differentiated and mesenchymal-like per se (25), we stimulated cells with retinoic acid, a neural differentiation inducer (26). Retinoic acid treatment successfully decreased the expression levels of the neural progenitor marker NANOG in nonsilenced cells, as

would be expected (RQ 1.42 ± SE 0.08 vs 0.48 ± SE 0.23 in retinoic acid-treated cells, p=0.0441). However, this reduction was not observed in shRNA1- and shRNA2-silenced cells; in fact, NANOG levels increased in shRNA1-silenced cells (RQ 0.41 ± SE 0.32 vs. 2.01 ± SE 0.69 in retinoic acid-treated cells, p<0.01) (Figure 5A). This finding suggests that the absence of the P2X7A isoform prevented neural differentiation of ACN cells, maintaining cells in an undifferentiated phenotype.

Retinoic acid treatment increased the vincristine resistance of cells lacking the P2X7A variant (Figure 5B), indicating that the absence of this isoform not only prevented neural differentiation but also enhanced resistance to vincristine, although not to doxorubicin (Figure 5B).

Cellular quiescence is a dormant-like cellular state that allows cells to evade cancer therapy, which largely targets proliferating cells (27). P2X7 receptor activation with γ-S-ATP, an ATP analog that is more resistant to hydrolysis, enriched

quiescent populations of ACN cells and consequently decreased those in G1 phase (Figures 5C, D). However, the presence of the selective P2X7 receptor antagonist A438079 reversed this effect in cells expressing the P2X7A isoform, while in shRNA1- and shRNA2-silenced cells, the effect was persistent (Figures 5C, D), suggesting a P2X7A-related role.
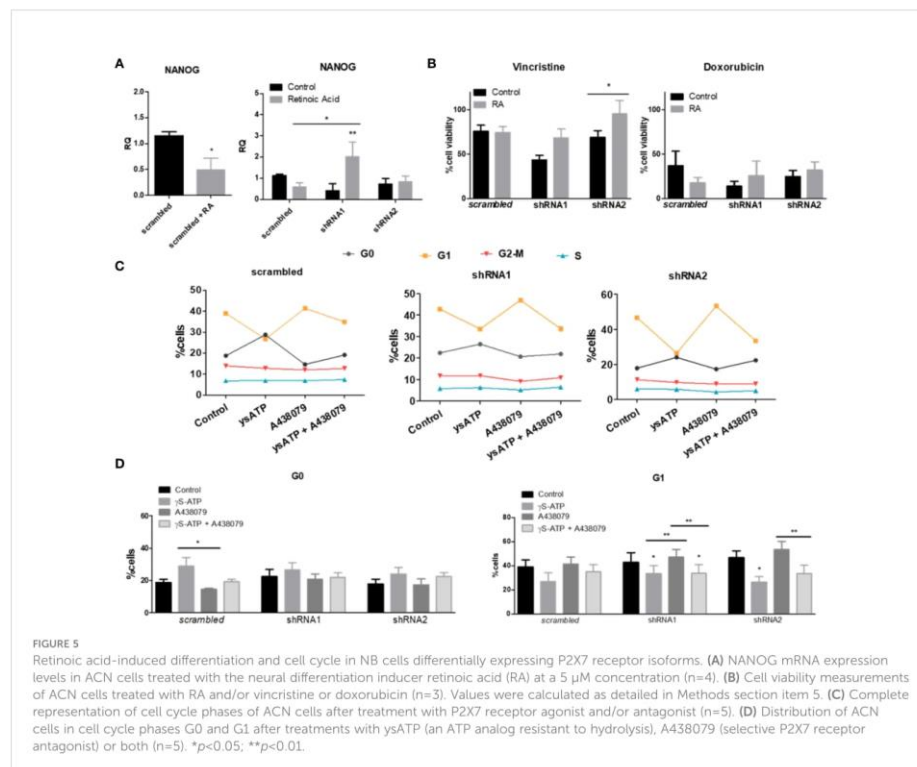
shRNA2-silenced cells exhibit decreased levels of autophagy markers, and its stimulation with rapamycin attenuates vincristine resistance, whereas starvation attenuates doxorubicin resistance
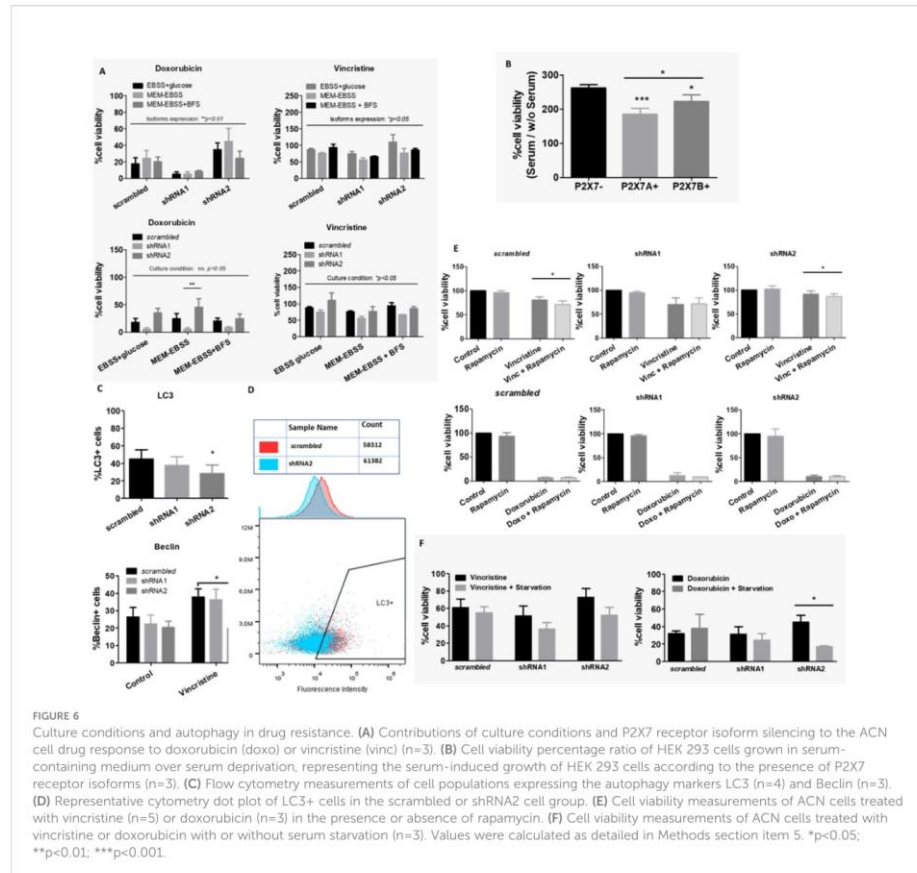
When testing culture conditions, we observed that ACN NB cells appeared to respond well to doxorubicin under most culture conditions, whereas vincristine resistance was severely impaired. The expression patterns of isoforms in ACN cells appeared to determine both vincristine and doxorubicin

responses (Figure 6A). Nonsilenced cells responded effectively to vincristine in EBSS-glucose and MEM-EBSS but not MEM-EBSS + FBS, pointing to a detrimental role of serum supplementation for drug efficacy when both P2X7 receptor isoforms are present, possibly indicating that growth factors and other molecules present in serum depend on the P2X7 receptor to induce proliferation or enhance cell survival. In shRNA1-silenced cells, drug response was better in MEM-EBSS and MEM-EBSS + FBS, reinforcing the hypothesis that the P2X7 receptor participates in the cellular response to serum compounds favoring drug resistance. Finally, shRNA2-silenced cells responded poorly under all conditions. For HEK 293 cells, culture conditions were the only determinant of doxorubicin effects (Figure S2B).

These differences suggest that metabolic pathways are essential for determining the drug resistance phenotype of both ACN and HEK 293 cells.

HEK 293 cells overexpressing P2X7A presented the lowest serum-induced growth ratio, followed by P2X7B-overexpressing

FIGURE 5
Retinoic acid-induced differentiation and cell cycle in NB cells differentially expressing P2X7 receptor isoforms. **(A)** NANOG mRNA expression levels in ACN cells treated with the neural differentiation inducer retinoic acid (RA) at a 5 μM concentration (n=4). **(B)** Cell viability measurements of ACN cells treated with RA and/or vincristine or doxorubicin (n=3). Values were calculated as detailed in Methods section item 5. **(C)** Complete representation of cell cycle phases of ACN cells after treatment with P2X7 receptor agonist and/or antagonist (n=5). **(D)** Distribution of ACN cells in cell cycle phases G0 and G1 after treatments with γsATP (an ATP analog resistant to hydrolysis), A438079 (selective P2X7 receptor antagonist) or both (n=5). *$p<0.05$; **$p<0.01$.

FIGURE 6

Culture conditions and autophagy in drug resistance. (A) Contributions of culture conditions and P2X7 receptor isoform silencing to the ACN cell drug response to doxorubicin (doxo) or vincristine (vinc) (n=3). (B) Cell viability percentage ratio of HEK 293 cells grown in serum-containing medium over serum deprivation, representing the serum-induced growth of HEK 293 cells according to the presence of P2X7 receptor isoforms (n=3). (C) Flow cytometry measurements of cell populations expressing the autophagy markers LC3 (n=4) and Beclin (n=3). (D) Representative cytometry dot plot of LC3+ cells in the scrambled or shRNA2 cell group. (E) Cell viability measurements of ACN cells treated with vincristine (n=5) or doxorubicin (n=3) in the presence or absence of rapamycin. (F) Cell viability measurements of ACN cells treated with vincristine or doxorubicin with or without serum starvation (n=3). Values were calculated as detailed in Methods section item 5. *p<0.05; **p<0.01; ***p<0.001.

cells, both of which were lower than *mock* cells ratio. This demonstrates that these cells can grow efficiently in the absence of serum, and as such, adding serum does not increase the growth efficiency as much (Figure 6B). The ability to grow in serum absence is a malignancy-related ability, as it allows cell survival in stressful situations. This is consistent with the increased drug resistance observed in P2X7 receptor-expressing NB cells, such as nonsilenced and shRNA2-silenced subtypes (Figure 1E).

Autophagy is tightly regulated and related to nutrient supply and stress stimuli. Therefore, we hypothesized that this important metabolic phenomenon might be involved in the observed drug resistance. To investigate this possibility, we measured autophagy markers in ACN cells and tested whether autophagy manipulation could modulate the drug response.

LC3 expression was decreased in shRNA2-silenced cells compared with the other cell groups, indicating low autophagic activity in cells that expressed isoform B only. In addition, Beclin-1 levels increased in control and shRNA1-silenced cells treated with vincristine, but this shift was not observed in shRNA2-silenced cells (Figures 6C, D). This finding suggests that shRNA2 cells do not exhibit increased autophagy in response to the stress stimuli induced by vincristine.

On the other hand, treatment with rapamycin, an autophagy inducer, decreased vincristine resistance in nonsilenced and shRNA2-silenced cells (Figure 6E). This finding indicates that compensation for the impaired autophagy observed in shRNA2 cells may be a strategy to attenuate drug resistance in this cell type. However, this was not the case for doxorubicin (Figure 6E).

When ACN cells were cultured under serum deprivation and in low glucose conditions, in comparison to high glucose plus serum supplementation, cell death rates in response to vincristine treatment increased independently of P2X7 receptor expression silencing (Figure 6F). However, for doxorubicin, this shift in cell death upon starvation was observed only in shRNA2-silenced cells (Figure 6F), suggesting that this attenuation in doxorubicin resistance may be related to starvation-induced autophagy upregulation.
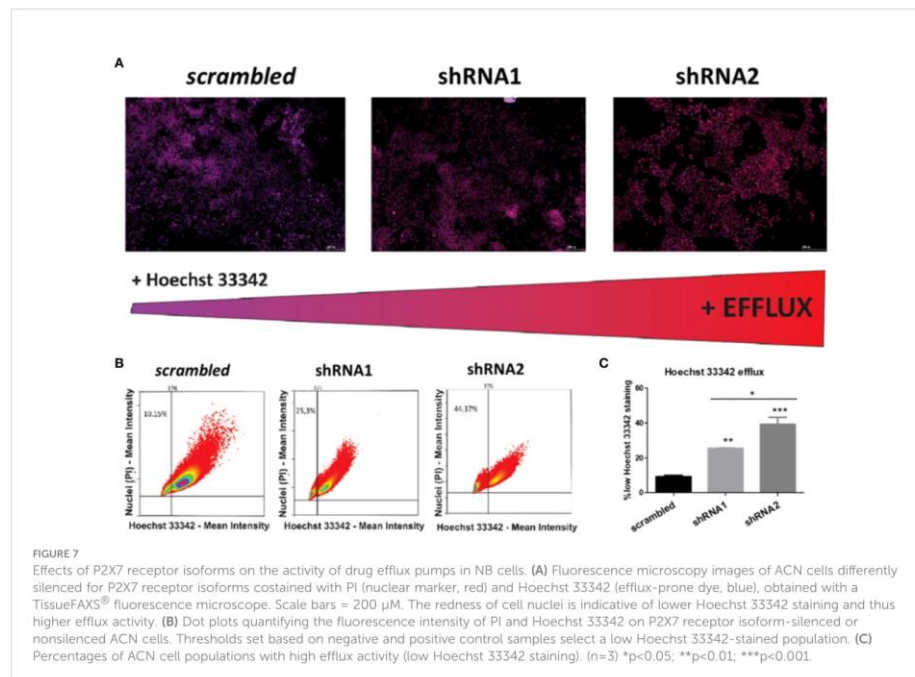
P2X7A has an efflux-preventing role, and P2X7B is associated with higher efflux activity, probably mediated by MRP-type ABC transporters

To investigate whether P2X7 receptor-mediated drug resistance is related to efflux pumps, an efflux activity assay was performed. shRNA2-silenced cells showed the highest efflux ability, followed by shRNA1-silenced cells (Figure 7). This suggests that P2X7A has an efflux-preventing role.

Although P-gp is the most expressed efflux pump among those verified in this study, MRP pumps appear to be the most relevant

for vincristine resistance in NB cells, as all ACN cell types exhibited increased MRP1 expression upon vincristine treatment (Figure 8A). In shRNA2-silenced cells, vincristine resistance was attenuated by MRP pump inhibition (probenecid) (Figure 8B), and Bliss independence analysis showed that probenecid acts synergistically with vincristine, reducing drug resistance (Figure 8C). P-gp inhibition only attenuated the drug resistance of shRNA1-silenced cells to doxorubicin, which does not seem to be a highly relevant resistance phenomenon because doxorubicin is highly effective in shRNA1-silenced cells (Figure S3). When the BCRP antagonist was administered concomitantly to anticancer drugs, no relevant differences were observed.

Upon EMT induction, HEK 293 *mock* cells raised MRP1 expression levels but decreased BCRP expression, and depended on ATP treatment to raise P-gp levels, an obviously not P2X7 receptor-mediated effect. When P2X7A was overexpressed, MRP1 and BCRP levels responded to EMT induction similarly to *mock* cells. However, P-gp levels were reduced, possibly due to P2X7A overexpression. In the case of P2X7B overexpression, the increase on MRP1 levels upon EMT induction depended on the presence of ATP (Figure 9), suggesting a critical role for the P2X7B variant in inducing MRP1 efflux pump expression, similar to the observed Twist2 expression patterns.

FIGURE 7
Effects of P2X7 receptor isoforms on the activity of drug efflux pumps in NB cells. **(A)** Fluorescence microscopy images of ACN cells differently silenced for P2X7 receptor isoforms costained with PI (nuclear marker, red) and Hoechst 33342 (efflux-prone dye, blue), obtained with a TissueFAXS® fluorescence microscope. Scale bars = 200 μM. The redness of cell nuclei is indicative of lower Hoechst 33342 staining and thus higher efflux activity. **(B)** Dot plots quantifying the fluorescence intensity of PI and Hoechst 33342 on P2X7 receptor isoform-silenced or nonsilenced ACN cells. Thresholds set based on negative and positive control samples select a low Hoechst 33342-stained population. **(C)** Percentages of ACN cell populations with high efflux activity (low Hoechst 33342 staining). (n=3) *p<0.05; **p<0.01; ***p<0.001.
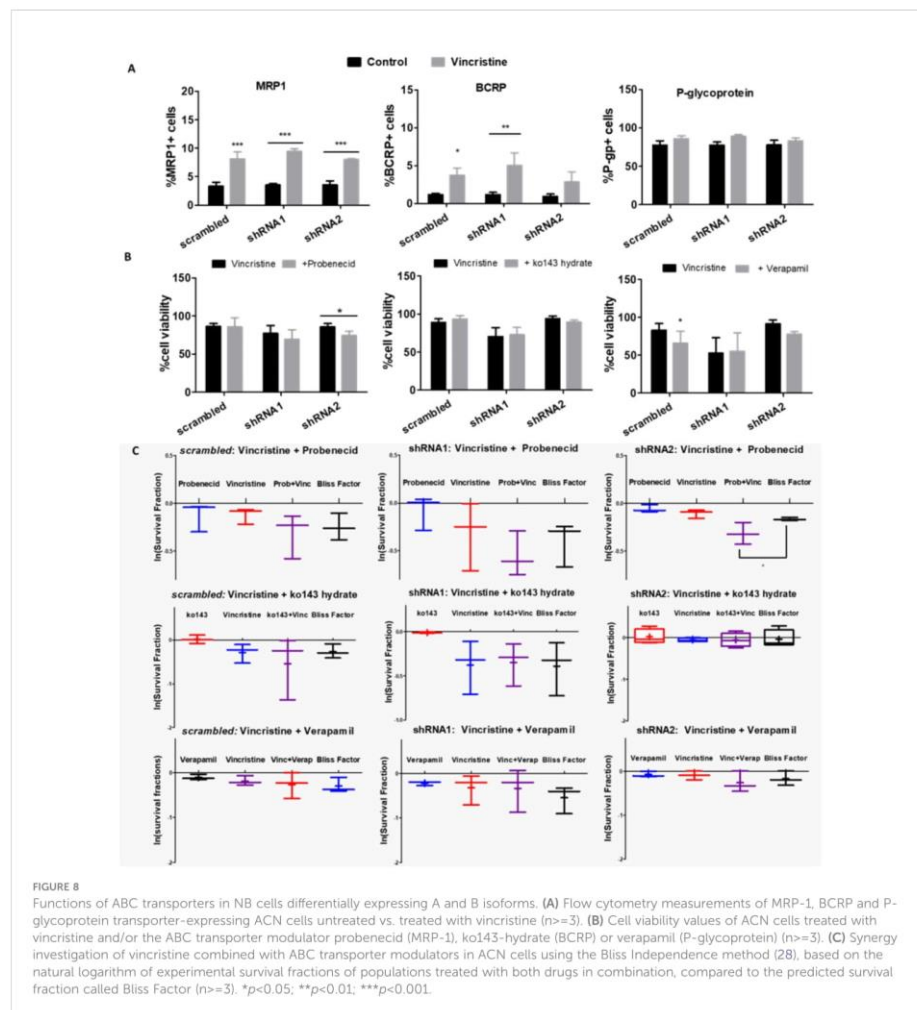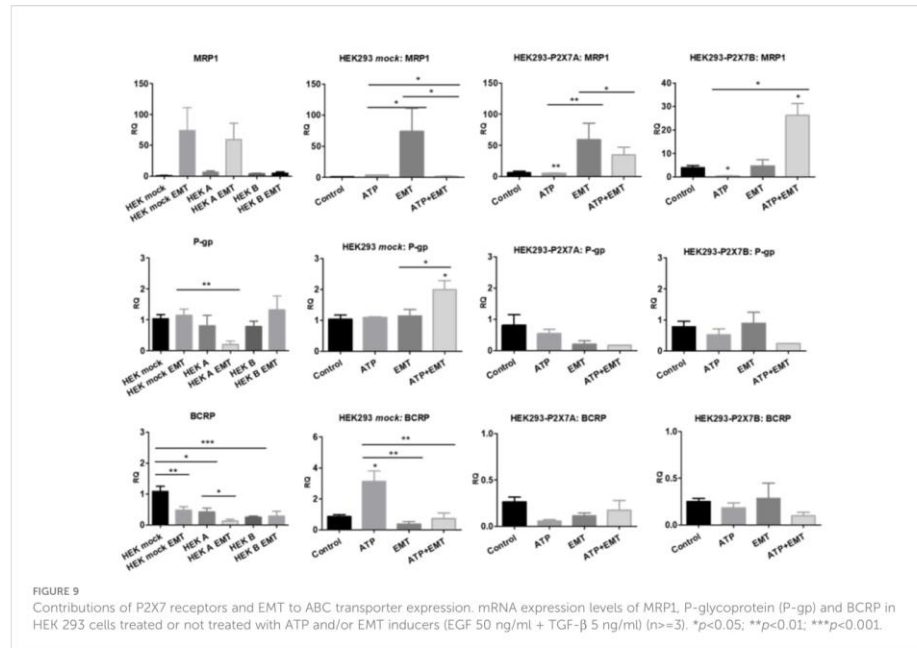
Discussion

The therapeutic combination of vincristine, doxorubicin, and cyclophosphamide constitutes one of the cycles of induction therapy widely used for the treatment of high-risk neuroblastoma (29). These drugs have complementary mechanisms of action, and the cytotoxicity of the combination is based mainly on targeting DNA (30). When used individually, their effects are expected to diverge in terms of potency and specificity, considering the differences between their mechanisms and properties.

In our study, we observed high resistance of NB cells to vincristine, which is a tubulin-binding compound that acts through microtubule disruption (30). Vincristine is efficiently effluxed from cells via P-gp, leading to strong resistance to vincristine treatment in cell types overexpressing this efflux

FIGURE 8
Functions of ABC transporters in NB cells differentially expressing A and B isoforms. **(A)** Flow cytometry measurements of MRP-1, BCRP and P-glycoprotein transporter-expressing ACN cells untreated vs. treated with vincristine (n>=3). **(B)** Cell viability values of ACN cells treated with vincristine and/or the ABC transporter modulator probenecid (MRP-1), ko143-hydrate (BCRP) or verapamil (P-glycoprotein) (n>=3). **(C)** Synergy investigation of vincristine combined with ABC transporter modulators in ACN cells using the Bliss Independence method (28), based on the natural logarithm of experimental survival fractions of populations treated with both drugs in combination, compared to the predicted survival fraction called Bliss Factor (n>=3). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

FIGURE 9
Contributions of P2X7 receptors and EMT to ABC transporter expression. mRNA expression levels of MRP1, P-glycoprotein (P-gp) and BCRP in HEK 293 cells treated or not treated with ATP and/or EMT inducers (EGF 50 ng/ml + TGF-β 5 ng/ml) (n>=3). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

pump (30). Indeed, we observed that >90% of the ACN NB-cell population expressed P-gp, regardless of P2X7 receptor isoform expression patterns.

The responses observed upon agonism or antagonism of the P2X7 receptor are consistent with previous findings. While the P2X7 receptor is widely known as a cell death-promoting receptor, absence of the complete C-terminal tail prevents macropore opening, compromising the ability to induce cell lysis (14, 15, 31, 32). Whenever both isoforms A and B are expressed, they tend to coassemble, forming a heterotrimeric receptor. In this particular setting, the B isoform may enhance the responses mediated by P2X7A (16). Additionally, P2X7B expression alone is the phenotype mostly related to growth promotion (18), supporting our evidence implicating P2X7B isoform importance in drug resistance. Consistently, we observed that P2X7A-expressing HEK 293 cells have higher death ratios in response to chemotherapy drugs. ACN cells with basal expression of the P2X7 receptor were more drug resistant than their counterparts silenced for both A and B isoforms, also corroborating the survival and proliferation functions previously observed for endogenous expression of the P2X7 receptor (15, 33). Recent studies have suggested a role for P2X7B in resistance to daunorubicin and radiotherapy, such that cancer treatments that increase the extracellular ATP levels in the TME may lead to

death of P2X7A-overexpressing cells while promoting the survival of cells overexpressing P2X7B (17, 34).
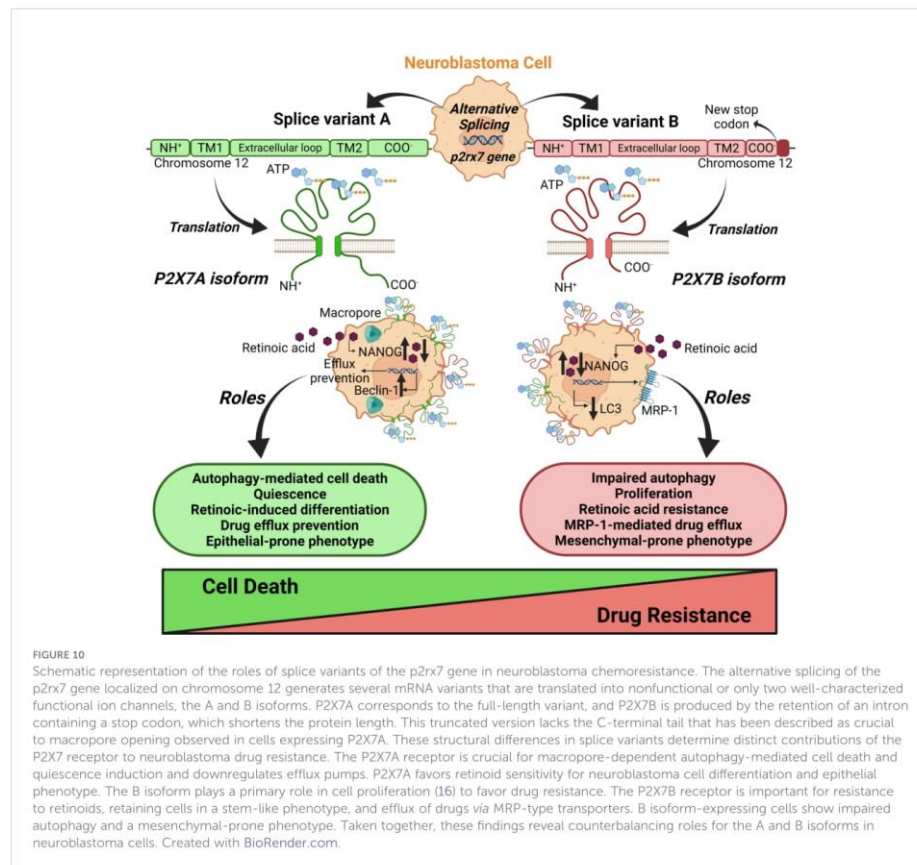
Here, we demonstrated that P2X7A is crucial for shifting the cell phenotype toward a more differentiated state, reducing cell stemness in response to retinoic acid. This finding is complementary to what was observed by Glaser *et al*: P2X7 receptor expression in embryonic cells was suppressed to allow neural differentiation (35). The most relevant isoform in this case is P2X7A, and when this isoform is absent in immature cells, differentiation is not triggered. Indeed, when used for neuroblastoma treatment, retinoic acid also faces resistance, a phenomenon termed retinoid resistance (36). If P2X7A expression relates to retinoid acid sensitivity, the detection of this isoform in the tumor mass might be a marker for guiding treatment decisions. Combining retinoic acid treatment with a P2X7 receptor agonist could also be a promising approach and is worth further investigation. Further preclinical studies could explore whether P2X7 receptor agonism together with retinoic therapy, prior to chemotherapy, would modulate the tumor mass toward a more drug-responsive phenotype, avoiding the selection of less differentiated resistant cells. Although there are still no specific agonists or antagonists for each isoform, both of them seem to be necessary for cell differentiation, meaning that a general agonist might work.

On the other hand, the P2X7B isoform was related to enhanced expression of EMT markers upon stimulation with well-known EMT inducers and increased EMT-triggered drug resistance. This finding is absolutely novel. Although relationships between EMT, invasiveness and the P2X7 receptor have been demonstrated (14, 37, 38), investigation of the involved isoforms has never been pursued. Because P2X7B is a main player in drug resistance and is also implicated in EMT, targeting this isoform in cancer seems promising for preventing EMT, thus overcoming drug resistance and tumor relapse.

shRNA2-silenced NB cells expressing only the P2X7B isoform showed decreased levels of autophagy markers. Induction of autophagy either pharmacologically or through starvation decreased resistance to the drugs used in our study,

suggesting that this autophagy impairment may be an actual contributor to NB-cell resistance. Although it is usually thought that autophagy would be cytoprotective and thus enhance resistance, autophagy triggering is a mechanism of inducing cell death. Indeed, enhanced sensitivity to doxorubicin when autophagy is inhibited has been reported for several cancers (39) and increased autophagy levels in response to a class I phosphatidylinositol 3 kinase/mTORC1 inhibitor enhanced doxorubicin-induced apoptosis of NB cells (40), consistently to our observations. Regarding vincristine, inhibition of autophagy is also generally beneficial to decrease cancer cell drug resistance (30).

Previous evidence points to the P2X7 receptor either as a positive or negative regulator of autophagy (14, 41–45). In one

FIGURE 10
Schematic representation of the roles of splice variants of the p2rx7 gene in neuroblastoma chemoresistance. The alternative splicing of the p2rx7 gene localized on chromosome 12 generates several mRNA variants that are translated into nonfunctional or only two well-characterized functional ion channels, the A and B isoforms. P2X7A corresponds to the full-length variant, and P2X7B is produced by the retention of an intron containing a stop codon, which shortens the protein length. This truncated version lacks the C-terminal tail that has been described as crucial to macropore opening observed in cells expressing P2X7A. These structural differences in splice variants determine distinct contributions of the P2X7 receptor to neuroblastoma drug resistance. The P2X7A receptor is crucial for macropore-dependent autophagy-mediated cell death and quiescence induction and downregulates efflux pumps. P2X7A favors retinoid sensitivity for neuroblastoma cell differentiation and epithelial phenotype. The B isoform plays a primary role in cell proliferation (16) to favor drug resistance. The P2X7B receptor is important for resistance to retinoids, retaining cells in a stem-like phenotype, and efflux of drugs via MRP-type transporters. B isoform-expressing cells show impaired autophagy and a mesenchymal-prone phenotype. Taken together, these findings reveal counterbalancing roles for the A and B isoforms in neuroblastoma cells. Created with BioRender.com.

study, P2X7 receptor agonism enhanced autophagic flux in a macropore-dependent way, leading to cell death, which was prevented by autophagy inhibition (42). In other words, the activation tonus of the receptor was decisive: while autophagy was increased upon tonic short-term stimulation with ATP, sustained stimulation with higher concentrations decreased autophagic flux (45). Once more, adding the isoforms to the equation helps clarify these divergences: while the P2X7A receptor is crucial to macropore-dependent autophagy-mediated cell death, as Fabbrizio et al. have shown, P2X7B receptor-expressing cells have lower autophagy levels and thus are less susceptible to autophagy-mediated cell death (Figure 10), as suggested by our data.

While P2X7A was important for preventing efflux, P2X7B related to the highest efflux phenotype in NB cells. This finding reinforces the perspective that for several functions, A and B isoforms have counterbalancing roles, as demonstrated for drug susceptibility, EMT, cell differentiation, and autophagy (Figure 10). This difference in efflux activity is most likely mediated by MRP-type pumps, as probenecid increased the susceptibility of P2X7B-expressing cells to vincristine. It has been demonstrated that treatment with retinoic acid decreases MRP1 expression levels in NB cells (46), which is consistent with our findings and helps conciliate all the evidence observed in the present work. If the absence of P2X7A maintains cells in a pluripotent state, as suggested by our data, and P2X7B expression increases drug resistance partly through MRP-type pump modulation, the inability of retinoic acid treatment to decrease pluripotency and thus MRP1 expression may be related to its inability to decrease drug resistance. In P2X7A variant-expressing cells, however, retinoic acid successfully decreased pluripotency and possibly MRP1 levels, as observed in the aforementioned study, explaining the reduced drug resistance.

Conclusion

Tumor malignancy depends on a complex setting of characteristics shared by its constituent cells. Cancer cell stemness, mesenchymal-epithelial grade, efflux activity, metabolic regulation, and crosstalk with stromal cells are just a few countless processes that influence tumor aggressiveness. Following the conclusion that the P2X7 receptor isoform expression pattern was critical for drug resistance of NB cells, we observed some of the main candidates that could explain P2X7B-mediated drug resistance. Our results point to cooperatively built drug resistance, which is the result of many aspects that individually present only moderate effects but together build up a relevantly resistant phenotype. Given the pleiotropic role of the P2X7 receptor in modulating various cellular functions, this is not surprising. On the one hand, we

demonstrated that P2X7A participates in triggering NB-cell differentiation, retinoid sensitivity and autophagy and downregulates efflux, and on the other hand, we showed the complementary role of P2X7B in suppressing autophagy, inducing drug efflux, and promoting EMT (Figure 10). The present work is thus highly relevant for resolving controversial findings in previous studies and proposing an integrated perspective to explore the P2X7 receptor.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

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Conflict of interest

HU is a scientific adviser of TissueGnostics, Vienna, Austria, and receives consulting fees.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.966404/full#supplementary-material

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The P2X7 Receptor in the Maintenance of Cancer Stem Cells, Chemoresistance and Metastasis

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Abstract
Metastasis is the worst prognosis predictor in the clinical course of cancer development. Features of metastatic cancer cells include migratory ability, low degree of differentiation, self-renewal and proliferation potentials, as well as resistance to therapies. Metastatic cells do not present all of the necessary characteristics at once. Indeed, they have a unique phenotypic plasticity, allowing the acquisition of features that make them successful in all steps of metastasis. Cancer stem cells (CSC), the most undifferentiated cells in the tumor mass, display highest metastatic potential and resistance to radio- and chemotherapy. Growing tumors exhibit marked upregulation of P2X7 receptor expression and secrete ATP. Since the P2X7 receptor plays an important role in the maintenance of undifferentiated state of pluripotent cells, its importance on cell fate regulation in the tumor mass is suggested. Considering the extensive crosstalk between CSCs, epithelial-mesenchymal transition, drug resistance and metastasis, current knowledge implicating P2X7 receptor function in these phenomena and new avenues for therapeutic strategies to control metastasis are reviewed.

Keywords Cancer stem cells · P2X7 receptor · Purinergic signaling · Metastasis · Drug resistance · Epithelial-mesenchymal transition

Introduction

Cancer is characterized by the disordered growth of cells that can spread to other parts of the body and invade specific tissues and organs to form secondary tumors, a process called metastasis. Metastatic disease accounted for 90% of tumor-related deaths 20 years ago [1] and despite large efforts to improve therapeutic outcomes, no robust improvements occurred regarding the survival of metastatic disease patients of most cancer types. [2–6] The heterogeneity of the tumor cell mass in solid tumors allows arising of metastatic cells as well as of cells resistant against current therapies, which are responsible for the main challenges in the clinical management of cancer patients. Notably, cells with an undifferentiated phenotype are more prone to survive chemo- and radiotherapy and have the ability to generate a whole new tumor, contributing to tumor recurrence and metastasis. These cells are denominated cancer stem cells (CSCs), able to self-renew or differentiate into non-CSCs. However, unlike normal cells, cancer cells present an unique plasticity and, therefore, they may dedifferentiate and contribute to CSC maintenance in tumor mass [7, 8]. Tumor phenotype alterations occur in response to environmental cues, mainly through epigenetic changes. Among the mechanisms coordinating cell plasticity, epithelial-mesenchymal transition (EMT) program is a central player in promoting reversible changes that affect migratory and invasive abilities of cells. In addition, EMT affects resistance against treatment, suggesting mechanistic links between CSCs, EMT and therapy resistance [9, 10]. As the degree of differentiation of tumor cells is strongly related to their aggressiveness and patients' prognosis [11, 12], the understanding of mechanisms that promote generation and maintenance of CSCs is fundamental for effective therapeutic strategies.

Purinergic signaling has been shown to regulate proliferation, differentiation, cell death and successful engraftment of stem cells originated from diverse origins [13]. The purinergic system is composed of nucleoside and nucleotide receptors [14]. P1 are G protein-coupled receptors responsive to adenosine. P2 receptors are either ionotropic receptors activated by ATP (P2X) or metabotropic/G protein-coupled receptors stimulated by ATP, ADP, UTP, UDP or UDP-glucose (P2Y).

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Seven P2X subunits are known so far, ranging from P2X1 to P2X7. Each P2X ion-channel is composed of three subunits, either in homo- or heterotrimeric compositions [15]. Among the purinergic receptors, P2X7 is the most widely expressed in tumor cell lineages and in human primary tumors [16]. Moreover, in tumor microenvironment (TME), extracellular ATP (eATP) concentrations can reach hundreds of micromoles per liter, in comparison to a nanomolar rate in healthy tissues [17, 18]. Taking into account the role of P2X7 receptor in the maintenance of the stemness state of embryonic cells [19], it might be involved in CSC plasticity, i.e., in the balance between differentiated and undifferentiated cancer cells within tumor bulk, and in favoring aggressiveness, tumorigenic potential and resistance to current therapies (Fig.1).

Dual Functions of P2X7 in Tumor Biology

Among P2X ion channels, the P2X7 receptor exhibits the lowest binding affinity to ATP, presenting an EC50 of ~ 300 μM [20]. This means that in the tumor microenvironment (TME), where eATP is markedly elevated, P2X7 receptor function is expected to be highly relevant. Solid tumors are tissues formed by cancer and normal stromal cells, including immune and mesenchymal cells that compose TME. Increased nucleotide and nucleoside concentrations within the TME are not simply a consequence of cell metabolism, but the evidence of a robust signaling mechanism regulating cancer development. They do not only modulate interactions between tumor and immune cells, but also several tumorigenic responses [16]. Intriguingly, the P2X7 receptor may have bifunctional roles under pathophysiological conditions such as cancer: its overstimulation induces tumor cell death while its tonic activation promotes tumor growth [21]. Being expressed both in cancer and stromal cells, P2X7 receptor roles in cancer biology are abundant.

P2X7 receptor roles in inflammation are very well established. In tumor host, the presence and function of this receptor is crucial for an effective antitumor immune response. Both genetic deletion and pharmacological inhibition of P2X7 receptor increase tumor incidence [22], as well as accelerate tumor progression in mice [23], effects accompanied by an extensive decrease in immune cells infiltration within tumor mass, reduction of pro-inflammatory mediators, involved in P2X7 receptor-mediated cell death, and elevation of anti-inflammatory cytokines [22, 23]. In a recent study, P2X7 receptor null mice bearing P2X7 receptor-expressing tumors developed an overall immunosuppressive TME, with higher regulatory T (Treg) cell infiltration, OX40 and CD73 overexpression and lower eATP levels, while wild-type mice that received treatment with a P2X7 receptor antagonist presented a small-sized tumor with higher TCD4+ lymphocyte infiltration and decreased expression of ectonucleotidases [24].

Furthermore, P2X7 receptor expression levels are upregulated in tumors and are understood as a bad prognosis predictor for several cancer types [25–30]. In addition, P2X7 receptor antagonism leads to tumor regression in animal models [21, 31]. Besides the known pro-inflammatory roles of
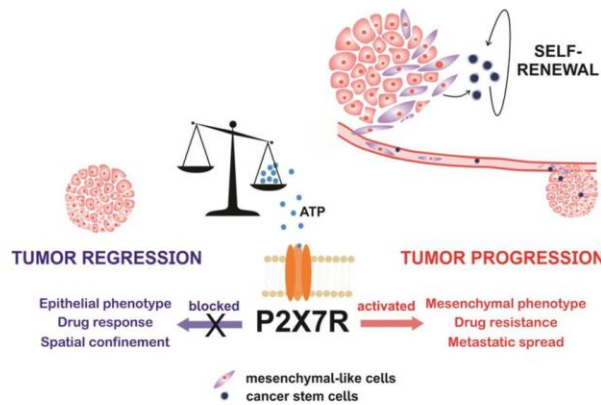

Fig. 1 P2X7 receptor functions in tumor progression and metastasis. ATP-gated P2X7 receptor is highly expressed in many tumor types. P2X7 receptor activation leads to tumor progression and metastasis, since it may induce phenotypic changes driving cancer cells to a stemness state, known as Cancer Stem Cells. These cells are able to promote tumor relapse following unsuccessful treatments, thanks to their self-renewal potential. Moreover, cancer cells may undergo epithelial-mesenchymal transition that increase metastatic capacity, and make them more resistant to chemo- and radiotherapy. Therefore, P2X7 receptor blockade may keep cancer cells in a differentiated epithelial state, being less aggressive and more treatable with available current drugs

P2X7 receptor [32–34], its expression also occur in anti-inflammatory cells, as myeloid-derived suppressor cells (MDSCs) [35], Tregs [36] and M2 macrophages [37], and relates to anti-inflammatory secretomes that may lead to tumor escape from the immune system [35, 37, 38].

These opposing roles regarding P2X7 receptor in cancer biology can be partially explained by different responses triggered by distinct levels of ATP stimulation. Besides that, the generation of distinct splice variants of the P2X7 receptor is also a way to regulate cellular responses to eATP. The human P2X7 subunit specifically presents as much as ten different isoforms (P2X7A-J), resulting from alternative splicing of the 13 exons comprising the human *p2rx7* gene [39, 40]. The well-characterized isoform is the full-length one, named P2X7A. Unlike P2X7A, which presents the longest cytoplasmic C-terminal tail, P2XB, P2X7E, P2X7G and P2X7J are truncated isoforms [41]. Among them, P2X7B is the only subunit that assembles a functional ion-channel. However, this isoform does not have the ability to form a large pore (LP) in the plasma membrane, related to cytolytic function and dye uptake observed in P2X7A receptors [42]. The inclusion of an intron between exons 10 and 11 inserts a stop codon that prevents translation of the full-length C-terminal tail in P2X7B. The deletion of TM1 by the insertion of a new exon, which is termed N3, introduces a start codon between exons 2 and 3, generating P2X7G and P2X7H isoforms, which are not able to assemble a functional receptor. Exclusion of exons in the extracellular loop induces structural alterations and functional ablation in P2X7C to P2X7F isoforms. The P2X7J isoform, truncated downstream of exon 7, is also non-functional and exerts a dominant negative role, neutralizing other isoforms by heteromerization. In cervical cancer cells, the P2X7J isoform is thought to protect cancer cells from P2X7 receptor-triggered apoptosis [43]. Post-translational modifications may also regulate receptor function. Phosphorylation of residue 343, for instance, inactivates the receptor [44], whilst N-linked glycosylation in some residues is crucial for receptor trafficking to the cell membrane [45].

Finally, the P2X7B isoform, which shares similar pharmacological properties with P2X7A [42], is not capable of inducing tumor cell death, but retains the ability to promote proliferation, showing that different isoforms also may give distinct contributions to tumor biology [41, 42], which may include the induction of cancer cells to chemoresistance and maintenance of CSCs in the tumor mass.

P2X7 Receptor and its Isoforms in the Maintenance of Cancer Stem-Cells (CSCs)

CSC's origin has been an issue of debate. There is a hypothesis that they derive from stem cells present in adult tissues, which are responsible for regeneration and tissue repair. The characterization of very small embryonic stem cells (VSELs) in several adult tissues and the subsequent confirmation of their stem cells' features strengthened the hypothesis of cell transformation into undifferentiated cells belonging to a back-up pool [46].

Another alternative for CSC origin consists in the de-differentiation of already transformed cells. While normal stem cells display an unidirectional differentiation way under physiological conditions, with fully differentiated cells being unable go back to an undifferentiated state [47], studies about cancer cell plasticity have shown that cancer cells have a fluctuating phenotype [7], which means they may be able to re-acquire stemness, reversing their differentiation fate. Thus, stemness is not a stable phenotype in the tumor mass; in contrast, a small subpopulation in the tumor can acquire stem-like cells proprieties depending on the microenvironment. Solid tumors are tissues formed by cancer and normal stromal cells, including immune and mesenchymal cells that compose the tumor microenvironment. In view of that, the niche in which CSCs reside finely regulates their identity, state and activity [48].

These cells are believed to be capable of promoting tumor recurrence and relapse following unsuccessful conventional treatments due to their self-renewal capability, in other words, their ability to differentiate into the various cell types of an heterogeneous tumor mass [11, 49] – including vascular endothelial cells, responsible for angiogenesis [50]. Once conventional treatments act eradicating proliferative tumor cells, a small subpopulation of cells within the TME can acquire drug tolerance, entering dormancy and later becoming resistant. These cells may also be called drug-tolerant persisters (DTPs) and evolve from self-renewal abilities to resist drug therapies [8].

For now, besides being upregulated in several tumor types, P2X7 receptor expression profiles are also associated to the degree of cell differentiation. Therefore, P2X7 receptor presents higher expression and activity in embryonic stem cells when compared to differentiated neural cells [19]. Accordingly, as the degree of cellular compromise with a given lineage increases together with the decrease of pluripotency markers, P2X7 receptor expression and activity are also suppressed. More interestingly, quiescent cells are induced to differentiate upon pharmacological inhibition of P2X7 receptor [19, 51].

Although the P2X7 receptor supposedly favors cancer survival, some findings have raised important questions about how its isoforms may correlate to the balance between cell death and survival, tumor arrest and progression, differentiation and de-differentiation of cancer cells. After sustained stimulation of P2X7 receptor in glioblastoma multiforme stem-like cells derived from primary human tumors, CSCs proliferation was decreased, which was attributed to cell death

and growth arrest [52]. Although cell death occurs in response to P2X7 receptor activation, growth arrest may induce surviving cells to enter a quiescent state, favoring chemoresistance and future tumor recurrence. Indeed, two weeks after the end of the treatment, cell regrowth as well as sphere formation were observed [52].

Again, different P2X7 isoforms may also contribute to controversial findings in different models. The role of the P2X7B isoform in pathophysiological conditions is not fully understood. Nevertheless, retention of cell growth promoting activity and loss of apoptotic function by this isoform has led to believe that its expression is more required than that of the P2X7A isoform in oncogenesis and maintenance of CSCs. Both isoforms (A and B) are extensively distributed among different tissues and usually appear assembled in the same trimeric receptor [42]. Coexpression of both isoforms potentiates traditional functions classically attributed to P2X7 receptor, including cell proliferation and invasion, in comparison to expression of the A isoform – except apoptotic function [42]. In osteosarcoma the B isoform alone is implicated to augment tumor cell density, reduce extracellular matrix deposition, as well as to promote Ca^{2+} mobilization, NFATc1 activity and cell cycle progress [41]. Treatment of neuroblastoma cells with bradykinin induced formation of metastases through the upregulation of P2X7B receptor expression, which was drastically reduced after pharmacological blockade of kinin-B2 receptor activity [53]. Together, these features seem to point to a more tumor-promoting role of the B isoform, involving extracellular matrix depletion, which is consistent with de-differentiation of the tumor. However, this idea still needs further experimental support.

Functions of P2X7 Receptor and Epithelial-Mesenchymal Transition (EMT)

Some of the characteristics described for CSCs are also shared by cells that undergo epithelial-mesenchymal transition (EMT) [54, 55], a trans-differentiation process that produces intermediate or partial phenotypes comprised in a spectrum that goes from epithelial to mesenchymal states, thus contributing to the heterogeneity of the tumor mass [56–58]. The EMT process occurring under physiological conditions, such as during embryonic development and tissue repair processes, is also important during pathogenesis [59, 60].

During EMT, epithelial cells lose their apical-basal polarity and their intercellular junctions, undergo cytoskeleton rearrangements and acquire mesenchymal features, including elongated morphology and increased migration and invasion capacity [61, 62]. Activation of transcription factors of the SNAI, ZEB, and TWIST families coordinates signaling pathways mediating these changes [63]. Cytoskeletal rearrangements include the replacement of cytokeratins intermediate

filaments by vimentin [64], and mesenchymal-like cells start producing enzymes that digest extracellular matrix components [65]. Moreover, proteins involved in epithelial cell-cell junctions, such as E-cadherin, are lost, and other adhesion molecules, such as N-cadherin and cadherin-11, become expressed [66]. These differences between epithelial and mesenchymal scenarios allow the detection of these components as molecular markers of EMT.

EMT renders tumor cells more motile and migratory [67, 68], meaning that the capability of undergoing EMT and acquiring a more mesenchymal-like phenotype may transform cells into colony founders guiding the metastatic process. However, whether EMT is required for metastasis is under debate. As EMT is a transitory phenomenon, it is challenging to track it in vivo [69]. Furthermore, characteristics that positively account for local invasion and intravasation are counterproductive for the metastatic seeding per se, calling for the EMT reversal, mesenchymal-to-epithelial transition (MET), to again make cells suitable for colonization [70]. It is also becoming widely recognized that epithelial cancer cells do not completely reverse their phenotype. They actually fluctuate among intermediate phenotypes. In view of that most research approaches may be considered somehow artificial [67, 69].

Two key experiments rekindled the debate after demonstrating that the deletion of individual EMT-inducing transcription factors failed to reduce metastasis in vivo [71, 72], and that secondary tumors were established mostly by epithelial cells that did not undergo EMT, as shown by the fluorescent tracking of cells that transiently expressed mesenchymal genes throughout metastatic process [72]. Conflicting evidence, however, implicates EMT in migratory [67] and invasive [73–75] abilities of cancer cells, and in metastatic spread per se [70, 75–77] in several highly relevant experimental setups. As such EMT participation in metastasis cannot be ruled out, although it might be not crucial. In agreement, other metastasis mechanisms coexist [78, 79]. Circulating tumor cells (CTCs) are both epithelial, mesenchymal, and with intermediate phenotypes, while more mesenchymal ones are related to disease progression [80]. Most importantly, EMT drives drug resistance [71, 72]. Being implicated both in metastasis and chemoresistance, EMT is likely a central phenomenon coordinating cancer recurrence.

Cells that undergo EMT are not necessarily stem cells, although they may acquire a similar phenotype and are related to a lower degree of differentiation [54, 55], greater metastatic capacity, chemoresistance and poor prognosis, similarly to CSCs [81–83]. In fact, stemness in not linked to a mesenchymal or epithelial state itself [84], but the triggering of an EMT program experimentally relates to the development of CSC properties [54, 55], although the observations are variable [85]. In breast cancer samples, for instance, CSCs have been characterized either as epithelial- or mesenchymal-like, and different pluripotency markers and localization in the tumor

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were characteristic of each population [84]. In addition, stemness potential is also associated with partial-EMT phenotypes, and the existence of an stemness window inside the EMT spectrum has been proposed, that would allow a fine-tuning of both properties in cancer cells [86]. In a non-cancerous context, EMT may be a phenomenon driving cells into more committed and differentiated stages, such as occurring during differentiation of pluripotent stem cells into mesenchymal stem cells [87]. CSCs, tumor-initiating cells (TICs) and cells with plastic EMT-MET phenotypes are conceptually interrelated, participating in the stemness character and in the heterogeneity of the tumor mass, conferring properties of chemoresistance and relapse potential to the tumor and favoring the appearance of metastatic disease. Thus, these cells represent the main challenge in current clinical practice.

Although the underlying mechanisms are not yet known, the hypothesis that overexpression of P2X7 receptor contributes to the maintenance of undifferentiated cells in the tumor mass and to the EMT phenotype seems reasonable, since CSCs and stem-like cancer cells have been widely implicated in the aggressiveness of tumors and in the metastatic processes.

Accordingly, experimental evidence implicates P2X7 receptor functions in cancer cell migration and invasion in several tumor types. In pancreatic ductal adenocarcinoma cells, the P2X7 receptor participates in the regulation of migration and invasion, responses directly associated with EMT [88]. Conversely, the P2X7 receptor agonist BzATP induced TGF-β1-independent EMT in renal tubular epithelial cells, resulting in differentiation for myofibroblasts. Further, differentiation stimulation into myofibroblasts, independently from TGF-β1 treatment, produced a similar upregulation in EMT-related genes than that promoted by the well-characterized EMT inducer TGF- β1. The observed effects were mediated by ERK 1/2 phosphorylation and reversed by the P2X7 antagonist OxATP [89]. In addition, P2X7 receptor expression silencing inhibited migration and invasion of prostate cancer cells in vitro and in vivo and attenuated the expression of genes involved in EMT. ERK 1/2 as well as the PI3K/Akt pathway phosphorylation are part of the P2X7 receptor-induced signal transduction cascade [90]. In breast cancer cells, hypoxia-induced EMT downregulated P2X7 receptor and other purinergic receptors expression and generated an overall reduction in sensitivity towards stimulation by ATP [91]. As previously reported, stimulation of P2X7 receptor enhanced PI3K/Akt activity and ERK1/2 phosphorylation, decreased GSK3β activity, activated the Akt/HIF1α axis and increased VEGF levels, which all are cellular events involved in the triggering of the EMT program [31, 92–94]. The involvement of P2X7 receptor in signaling pathways described for EMT inducers points at this receptor as a promising target for therapeutic prevention or reversal of EMT.

Furthermore, in many cell types, including macrophages and human tumor cells, P2X7 receptor stimulation drives the secretion of MMP2, a metalloprotease involved in EMT activation [95, 96]. This finding points at another possible mechanism of P2X7 receptor-mediated EMT induction that may happen through stromal cell signaling or direct cancer cell communication. Importantly, MMP2 cleaves P2X7 receptor following its secretion, creating a receptor modulation pathway that may help cancer cells to evade P2X7 receptor-triggered cytolytic actions. Therefore, besides the expression of P2X7 receptor isoforms unable to open a cytolytic macropore, other events downstream of P2X7 receptor activation may participate on cancer cell evasion of apoptosis. Coordination of phenotype plasticity through modulation of cell cycle, differentiation fate decision and EMT may drive tumor cells more aggressive and drug-tolerant the more undifferentiated and, in most cases, the more mesenchymal-like the phenotype is. Therefore, the modulation of P2X7 activity may bring important benefits for patients with cancer, for instance, preventing both evasion of P2X7 receptor-induced apoptosis and EMT, an important process for tumor progression and recurrence, chemoresistance and metastasis (Fig. 2).

Relations between P2X7 Receptor and Drug Resistance

Resistance to therapies, either intrinsic or acquired, is a growing problem in cancer management. Anything that reduces drug availability near its target or decreases the cell ability to respond to it will confer therapeutic resistance. Mainly, chemoresistance happens by either pharmacokinetic and metabolic changes or apoptosis and autophagy deregulation [97].

Autophagy is a phenomenon that physiologically works to protect cells by degrading damaged or not useful cellular products, thus generating byproducts that can function either as energy sources or as building blocks for new molecules [98, 99]. In cancer, autophagy may help starved and hypoxic cells distant from the blood stream to obtain ATP and survive, being an important tumor progression enhancer [100–102]. Moreover, autophagy protects tumor cells from several stressor agents, and its inhibition has been described to sensitize cancer cells to treatments and enhance cell death [103–107]. In addition, evidences have pointed that autophagy is necessary for stemness and tumorigenicity of populations with cancer stem cell properties [108, 109], and disruption of autophagy homeostasis consistently favors cell differentiation [110]. At a first look, autophagy may be considered pro-tumoral, since it increases malignant features in cancer cells and support tumor progression [111–113]. However, it is protective against tumor initiation because of its roles in maintaining genomic integrity, as well as regulating oxidative stress [114–116]. Furthermore, autophagy may either favor cell

Fig. 2 Relations between P2X7 receptor activation, EMT and stemness state, resistance to therapy and metastatic potential. P2X7 receptor is upregulated in many tumors and ATP levels are high in the tumor microenvironment. Both ATP-gated P2X7 receptor opening and epithelial-mesenchymal transition (EMT) induction trigger PI3K/Akt and ERK1/2 signaling pathways. The EMT process produces intermediate phenotypes with distinct degrees of differentiation. Cancer cells may lose their apical-basal polarity and acquire mesenchymal features, including elongated morphology, or vice-versa. The more undifferentiated the cancer cells, the more aggressive and invasive they are, with enhanced stemness, chemoresistance, and metastatic potential
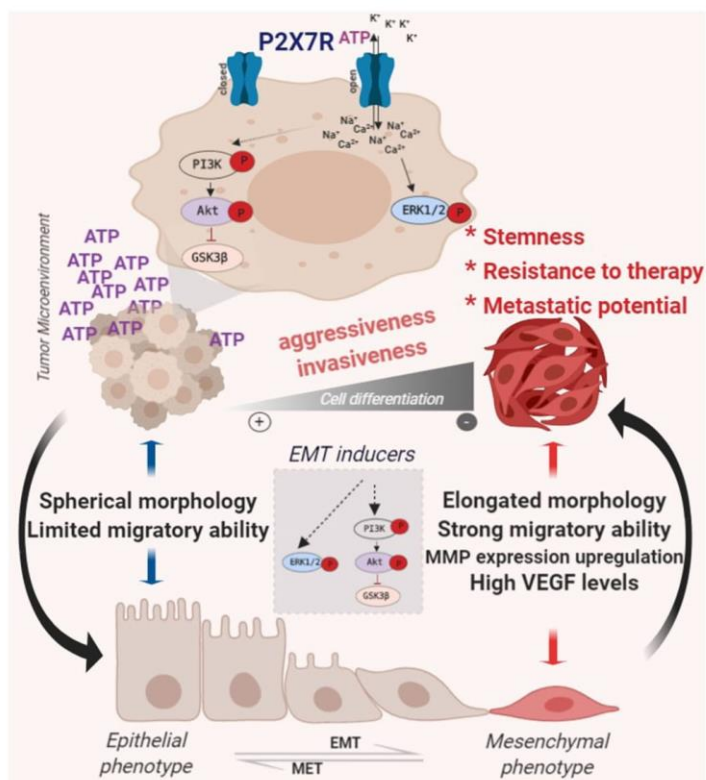
survival or induce death [117–119], which suggests that its roles in chemoresistance may be paradoxal as well.

The P2X7 receptor was suggested to be a positive regulator of autophagy in a traumatic brain injury model [120]. In dystrophic mice myoblasts, P2X7 receptor activation increased autophagic flux through large pore (LP) formation, culminating in cell death. In these cells, autophagy blockade inhibited ATP-dependent cell death, pointing at an autophagy-mediated and LP formation-dependent ATP-induced cell death [121]. However, these observations were not reproduced in macrophages [121], although studies suggest that P2X7 receptor-mediated autophagy is an important mechanism for mycobacteria killing [122, 123]. In other models, P2X7 receptor activation actually decreased autophagy as in mouse microglia [124, 125]. Furthermore, in primary microglia of an ALS mouse model, the intensity of receptor activation was determining for P2X7 receptor-mediated response: while short-term moderate ATP stimulation increased autophagy,

sustained stimulation with high concentrations negatively modulated autophagic flux [126]. This also brings up questions about different P2X7 receptor isoform participation in opposing responses.

These findings provide a mechanistic link between P2X7 receptor and autophagy, raising outstanding questions about whether or how P2X7 receptor could modulate autophagy in cancer cells, and whether it would drive quiescence and drug resistance or stop triggering cell death.

ATP-binging-cassette (ABC) transporters consist of transmembrane proteins capable of translocating various substrates from intra- to extracellular medium, regulating the biodistribution of endogenous and exogenous products. Under physiological conditions, these transporters play roles in steroid synthesis, immunological responses and various reproductive functions acting on barriers, such as placenta and blood-testicular barriers [127]. Because of their efflux-promoting function, they are able to remove

certain drugs from the cell, decreasing their interaction with intracellular pharmacological targets and reducing therapeutic effects. These transporters are ATP-dependent and as such have at least one subunit with ATPase activity [128, 129]. In several cancers, ABC transporters overexpression, including P-gp (P-glycoprotein, or ABCB1), MRP1 (multidrug resistance protein 1, or ABCC1) and BCRP (breast cancer resistance protein, or ABCG2), comprise some of the main chemoresistance mechanisms.

Notably, CSCs often present higher expression of these transporters than differentiated tumor cells [130], suggesting a reason for enhanced chemoresistance. In neuroblastoma, retinoic acid treatment favors cell differentiation and reduces MRP1 expression [131], suggesting that the degree of differentiation may be related to efflux pumps expression and consequently chemoresistance. In human cancers-isolated cells, Liu et al. (2014) verified an elevation in ABCG2 expression in a stem-like side population following culture in high glucose conditions. These data suggest that these cells are prone to be more chemoresistant when nutrients' supply is abundant, probably due to activation of Akt signaling pathway for regulation of glucose homeostasis [132]. Importantly, the progressively emerging links between stemness, EMT and drug resistance constitute important evidence that therapy resistance is not only a consequence of natural selection of resistance-prone genes, but also a transient state related to cell plasticity and epigenetic modulation.

Possible relationships between P2X7 receptor and ABC transporters in the context of chemoresistance have not been studied to date. However, downstream events of P2X7 receptor activation may involve ABC transporters, as the release of IL-1β through ABC1 transporters [133]. Exocytic and conductive mechanisms mediate ATP release to the extracellular medium [134–139]. Among the conductive pathways, the P2X7 receptor is a very well-established one [140], and evidences implicate ABC transporters in facilitating ATP release [141–143], thus it is likely that interrelations between these components participate in the regulation of extracellular concentration of ATP within tumor microenvironment and the availability of ectonucleotidase substrates as well [129]. If there are relationships between P2X7 receptor activity, CSCs and chemoresistance, it is reasonable that the expression and function of efflux pumps is involved.

Enrichment of lipid rafts has been described in membranes of breast and prostate cancer cell lines [144]. Considering that the cholesterol present in cell membranes prevents truncated P2X7 receptor isoforms from opening the macropore, as recently demonstrated [145, 146], it is reasonable to hypothesize that cholesterol-rich lipid raft regions are privileged locations for P2X7B maintaining its ion channel functions only and evading apoptosis. Very interestingly, cholesterol depletion or lipid rafts disruption increases cancer cell death [144, 147].

Non-functional variants of P2X7 (nfP2X7), which expose a particular epitope in the extracellular loop region and are not able to form a membrane pore [148], are highly expressed in several tumor types [25, 149, 150]. Besides being upregulated in its expression rate upon exposure to high ATP concentrations, nfP2X7 is fundamental to cancer cell survival [151], and might as well influence drug resistance. Antibodies developed to recognize nfP2X7 (E200) target the 200–216 amino acid sequence [148, 152]. These antibodies are being used as biological drugs for basal cell carcinoma treatment and already underwent phase I clinical trials, proving to be safe and tolerable [152].

Final Remarks

The ability of a cancer cell to proliferate and metastasize, i.e., to spread from a primary to distant sites in the body, forming secondary tumors, depends on intrinsic factors and on the tumor microenvironment. Nevertheless, successfully reaching another location in the body does not guarantee that a metastatic tumor will be formed. Secondary tumor formation depends on further factors released into the metastatic niche, and on the ability of the cells to undergo phenotypic changes that allow adaptation to the new microenvironment, as previously discussed. These phenomena enable cells to generate a progeny of cells that are able to proliferate and acquire more differentiated characters leading to tumor progression in distant tissues.

Tumor relapse and metastasis after radio- or chemotherapy is a basic clinical problem [153, 154]. There is much evidence that dormant CSCs in the metastatic niche can be activated upon therapy. The reason for this would be an inflammatory molecules-induced pro-metastatic microenvironment generated by healthy cells, which are dying due to unspecific effects of the treatment [154, 155]. eATP is a molecule related to inflammation, acting via P2X7 receptor and further purinergic receptor subtypes [156, 157]. ATP levels in the bone marrow, a metastatic target of many tumor types, are increased in mimetic models of radio- and chemotherapy [158]. Furthermore, this nucleotide is a chemotactic agent for cancer cells, besides enhancing the chemotactic response to stromal cell-derived factor-1 (SDF-1) and synergistically acting with other pro-inflammatory molecules in order to induce metastasis [53]. Many efforts have been made to elucidate the mechanism of tumor metastasis to the bone marrow. Recently, our group reported that metastasis of neuroblastoma to the bone marrow is drastically inhibited by P2X7 receptor antagonism in a murine xenographic model. Most importantly, neuroblastoma cells became more metastatic when P2X7 receptor expression was increased [53]. Although the mechanism underlying the process of metastatic blockade through P2X7 receptor antagonism is not fully elucidated, several pieces of evidence suggest a role of the P2X7 receptor in the CSC maintenance in the

tumor mass. As conventional anti-cancer therapies do not eliminate CSCs, they may actually enrich the percentage of CSCs, which are endowed with greater metastatic potential. Although more research is needed to elucidate the roles of the P2X7 receptor and its splice variants in maintaining CSC content in the tumor, we have reviewed strong evidence on the potential of this receptor as a target for improving anti-cancer therapies (Fig. 1).

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Using Cytometry for Investigation of Purinergic Signaling in Tumor-Associated Macrophages

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● **Abstract**

Tumor-associated macrophages are widely recognized for their importance in guiding pro-tumoral or antitumoral responses. Mediating inflammation or immunosuppression, these cells support many key events in cancer progression: cell growth, chemotaxis, invasiveness, angiogenesis and cell death. The communication between cells in the tumor microenvironment strongly relies on the secretion and recognition of several molecules, including damage-associated molecular patterns (DAMPs), such as adenosine triphosphate (ATP). Extracellular ATP (eATP) and its degradation products act as signaling molecules and have extensively described roles in immune response and inflammation, as well as in cancer biology. These multiple functions highlight the purinergic system as a promising target to investigate the interplay between macrophages and cancer cells. Here, we reviewed purinergic signaling pathways connecting cancer cells and macrophages, a yet poorly investigated field. Finally, we present a new tool for the characterization of macrophage phenotype within the tumor. Image cytometry emerges as a cutting-edge tool, capable of providing a broad set of information on cell morphology, expression of specific markers, and its cellular or subcellular localization, preserving cell–cell interactions within the tumor section and providing high statistical strength in small-sized experiments. Thus, image cytometry allows deeper investigation of tumor heterogeneity and interactions between these cells. © 2020 International Society for Advancement of Cytometry

● **Key terms**

macrophage; TAM; cancer stem cells; purines; ATP; purinergic system; EMT; image cytometry

Immune cells are a major component of the tumor microenvironment (TME), with the most abundant and well-characterized cells being macrophages, which constitute up to 80% of the tumor mass (1, 2). Macrophages are phagocytic cells able to influence local and systemic functions in response to endogenous and exogenous stimuli, orchestrating inflammatory responses through the secretion of several molecules as cytokines and growth factors. Depending on the function and stage of maturation, macrophages acquire diverse morphologies and express specific subsets of bioactive molecules and surface markers (3–5).

Classical macrophage (M1) activation is triggered by pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) through Toll-like receptor 4 (TLR-4), or cytokines like tumor necrosis factor alpha (TNF-α) or interferon gamma (IFN-γ) produced by Th1 cells (6), in response to lesions or infections. Generated stimuli lead to the activation of transcription factors, such as signal transducer and activator of transcription (STAT)-1, STAT-2, and hypoxia inducible factor 1α (HIF-1α) and production of pro-inflammatory cytokines, like TNF-α, interleukin (IL)-1β, IL-6, IL-12, IL-18, and IL-23, and chemokines, as C-X-C chemokine 10 (CXCL10) (6–8). In addition, M1 macrophages produce high levels of reactive

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oxygen (ROS) and nitrogen species, such as nitric oxide (NO) (8), likely through the expression of inducible NO synthase (iNOS) in murine macrophages (7). Alternative macrophage polarization (M2) is mainly induced by IL-4 or IL-13 released by Th2 cells, activating the transcription factors STAT-6, interferon regulatory factor (IRF)-4 and HIF-2α (6,9). They produce anti-inflammatory cytokines, including IL-10, C–C chemokine 17 (CCL17) and CCL22. Some of these molecules are experimentally used as molecular markers of macrophage phenotypes, as shown in Table 1. However, the M1 and M2 terminology is not enough to define all possibilities of macrophage activation phenotypes (10).

Polarization status also influences the activation of inflammasomes, multiprotein complexes of well-known importance for innate immune response (11). Importantly, primary human macrophages polarized toward the M2 phenotype through treatments with IL-4 and IL-13 do not respond to LPS by inducing NOD-like receptor (NLR) pyrin-domain-containing 3 (NLRP3) inflammasome expression. Opposingly, macrophages treated with IFN-γ and further exposed to LPS effectively raised NLRP3 expression levels, as well as caspase-1 activity and IL-1β secretion, reinforcing the importance of the polarization state for mounting an effective immune response (12).

Tumor-associated macrophages (TAMs) play an essential carcinogenic role in tumor progression, including metastasis enhancement (2). Generally, TAMs are related to the M2 phenotype, although recent studies have also shown a pro-tumor function of M1 macrophages (13–16). Molecular markers for TAMs mostly consist of canonical macrophage markers that recognize this cell type among the tumor mass, and somewhat overlap M2 phenotype markers (Table 1). However, unlike the binary definition M1/M2, TAMs are composed of several distinct populations comprised within a spectrum between pro- and anti-inflammatory definitions, sharing characteristics with both phenotypes (17).

Among the consequences generated by tumor development there are some molecules called damage-associated molecular patterns (DAMPs), including calreticulin, adenosine triphosphate (ATP), high-mobility group box 1 (HMGB1), S100 proteins and heat-shock proteins (18). DAMPs are commonly associated to cell death and tissue injury, since the major signaling molecules are cellular compounds (19, 20). As DAMPs are a heterogeneous group of molecules, the intracellular signaling pathways triggered are also diverse and activate either TLRs, receptor for advanced glycation endproducts, or NLRs responses (19).

Several molecules, including extracellular purines, participate in signaling events in the tumor microenviroment (TME). Extracellular ATP (eATP) and its dephosphorylated form adenosine are the main agonists of the purinergic system, acting as extracellular paracrine and autocrine signaling molecules (21). High extracellular nucleotide levels are released in response to cell damage, hypoxia, or mechanical stress (22, 23). eATP activates ligand-gated ion channels, called P2X receptors (P2X1–7), and G protein-coupled P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11–14), while adenosine activates metabotropic P1 receptors, named A_1, A_{2A} and A_{2B}, and A_3 receptors (21).

Table 1. Compilation of molecular markers described in the literature for different macrophage phenotypes

PHENOTYPE	HUMAN MARKERS	MURINE MARKERS
M1-polarized	*Cytokines*: TNF-α, IL-1β, IL-6, IL-12, IL-23, CXCL9, CXCL10, CXCL11	*Cytokines*: TNF-α, IL-1β, IL-6, IL-12, IL-23, CXCL9, CXCL10, CXCL11
	Membrane epitopes: CD38, CD64, CD80, CD86, CCR7(CD197), HLA-DR(MHC-II)^{high}	*Membrane epitopes*: CD38, CD64, CD80, CD86, CCR7(CD197), MHC-II^{high}
	Enzymes: IDO	*Enzymes*: NOS2 (iNOS)
M2-polarized	*Cytokines*: IL-10^{high}, IL-4, TGF-β, CCL17, CCL18, CCL22, CCL24	*Cytokines*: IL-10^{high}, IL-4, TGF-β, CCL22
	Membrane epitopes: CD163, CD23, MRC1(CD206), CD204, CD301	*Membrane epitopes*: CD163, MRC1 (CD206), MARCO, CD301
	Enzymes: TGM2	*Enzymes*: Arginase-1, TGM2
		Transcription factors: Egr2, c-Myc
		Others: Fizz1, Ym1/2
Tumor-associated macrophages (TAMs)[a]	*Membrane epitopes*: CD14, CD68, CD16, CD312, CD115	*Membrane epitopes*: F4/80, CD11b, CSF-1R, CD115, Gr1$^-$, Ly6C, CX3CR1, CCR2, CD26L, Tie2
Metastasis-associated macrophages (MAMs)	*Membrane epitopes*: VEGFR1(FLT1)^{high}	*Membrane epitopes*: VEGFR1 (FLT1)^{high}, CCR2^{high}, CXCR4$^-$, Tie2$^-$, CD45^{high}CD11b^{high}, CD11c^{low}, Ly6C^{low}

[a]TAMs exhibit canonical macrophage markers plus specific subtype markers that characterize its phenotypes as M1- or M2-oriented (7,10,15,17,184–189).

Herein, we review the biological actions of P2X and P2Y receptors in macrophages along with their interrelations in the TME. We highlight how subpopulations of TAMs may promote tumor development, as well as how the purinergic signaling system is involved in this process. Furthermore, we present image cytometry as a technique to assess macrophage polarization within the tumor based on single cell analysis, while preserving cell–cell interactions in TME.

MACROPHAGES AND CANCER BIOLOGY

Purinergic Signaling in Cancer-Related Inflammation

Based on robust findings, the scientific community has consensually established that chronic inflammation may lead to cancer (24). The nonconversion of a pro-inflammatory state to an immune-regulated one creates a mutagenic and chronically inflammatory scenario favoring genetic instability, which is related to altered DNA methylation patterns and aberrant gene expression. However, defects in immune surveillance play a central role in cancer biology as well, being considered a novel cancer hallmark (25, 26). Thus, inflammation is either essential to favor tumor initiation or to block cancer development (24).

Several lines of evidence correlate pro-inflammatory events, such as the activation of nuclear factor kappa B (NF-κB) and STAT-3, leading to downstream pathways involving IL-1, IL-6, TNF-α, chemokines, and cyclooxygenase 2 (COX-2) and early carcinogenesis and tumor initiation. The lack of NF-κB checkpoints is also correlated to enhanced carcinogenesis (24).

Purinergic receptors expressed in diverse cell types including cancer, immune and surrounding tissue cells respond to

eATP released within sites of inflammation or injury. The activation of TLR-2 and TLR-4 in macrophages, for instance, drives ATP release through connexins (27) in a loop response, in which inflammatory signals lead to further increases in inflammation. In addition, eATP recruits immune cells, such as macrophages and phagocytes, neutrophils, and dendritic cells (DCs), to the inflammatory site (28) through activation of purinergic receptors, such as P2X7 and P2Y2 subtypes (29). These receptors mediate pro-inflammatory events; that is, upon LPS stimulation, P2Y2 receptor expression is upregulated, potentiating pro-inflammatory signaling through eATP (30). P2X7 receptor activation is associated to NLRP3 inflammasome assembly in the M1, but not in the M2 macrophage subpopulation, leading to the release of inflammatory cytokines, including IL-1β (Fig. 1) (31). Evidence implicates IL-1β in HIF-1α-mediated oncogenesis (32). ATP-triggered DC migration is also mediated by P2X7 receptor as well as leukocyte recruitment (28, 33). Furthermore, P2X7 receptor activity reduces T-regulatory cell-mediated immunosuppressive effects and favors Th17 polarization of T lymphocytes, thus enhancing inflammation (34). These findings reinforce P2X7 receptor roles not only in innate, but also in adaptive immunity. Furthermore, P2X7 receptor activation is related to cytotoxicity (35) and participates in host response to pathogens (34), raising the hypothesis that it may be a key regulator of host defense against tumor cells as well. Activation of macrophages TLRs regulates the transcription of cytokine precursors through NF-κB signaling, while ATP-triggered P2X7 receptors activate caspase-1, allowing the cleavage and secretion of mature IL-1β and IL-18 (Fig. 1) (36). Thus, P2X7 receptor activation drives the production of reactive nitrogen and oxygen species
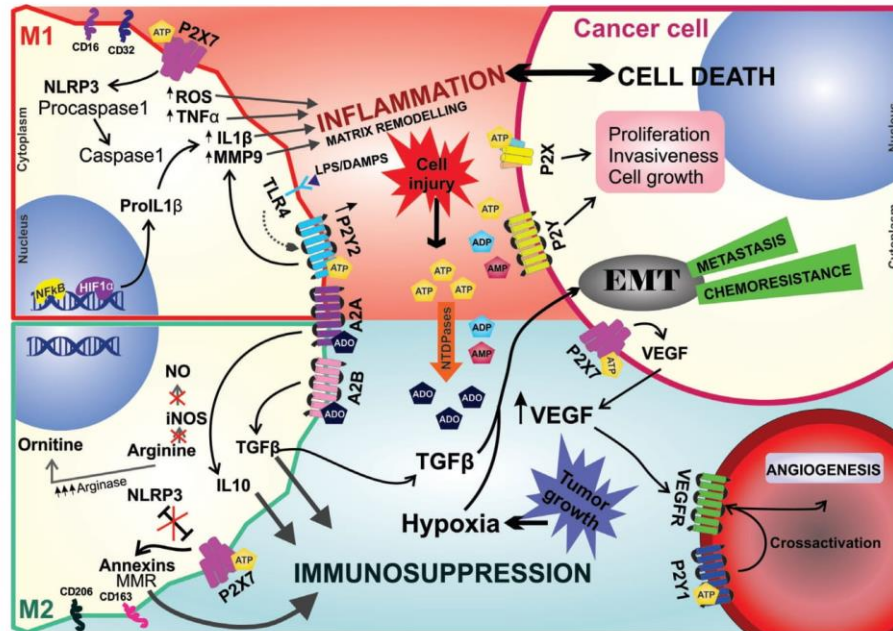
Figure 1. Purinergic signaling in the crosstalk between macrophages and the tumor microenvironment. Activation of purinergic receptors may drive macrophage polarization and lead to the secretion of pro- or anti-inflammatory compounds, triggering either cancer cell death or tumor progression. In several cases, inflammasomes mediate these events, triggered by ATP either through P2X7 or P2Y2 receptors. Environmental cues, like hypoxia, modulate the activity of ectonucleotidases, regulating the availability of adenosine (ADO) for action on A2A or A2B receptors, which drives immunosuppressive responses. Activation of purinergic receptors in cancer cells is important for key events of tumor progression, like chemotaxis, epithelial-to-mesenchymal transition (EMT), invasiveness and proliferation. [Color figure can be viewed at wileyonlinelibrary.com]

and pro-inflammatory lipids, and augments TNF-α release due to raised extracellular activity of TNF-α-converting enzyme (TACE) (37).

Accordingly, several studies confirmed that the P2X7 receptor plays a role in host antitumor response. Adinolfi et al. reported the increase of tumor progression and metastasis in P2X7 receptor knockout mice (P2X7$^{-/-}$) after injection of B16 melanoma and CT26 colon carcinoma cells (38). Hofman et al. also reported an increase in tumor development after inactivation of P2X7 receptors in a colitis-associated cancer mouse model, along with reduced inflammation (39).

However, evidence that inflammation-related features, such as elevated ROS levels, IL-1β, TNF-α and TACE, are related to tumor progression and metastasis (40, 41) may suggest a pro-tumoral role of both P2Y2 and P2X7 receptors, meaning that the inflammation promoted by these receptors may actually be deleterious in some stages of tumor development, rather than exclusively beneficial. In fact, in breast

cancer cells, the P2Y2 receptor was involved in inflammasome assembly, culminating in secretion of pro-inflammatory cytokines and several pro-tumoral responses, such as cell invasion (42). NLRP3 assembly in response to ATP stimuli through the P2X7 receptor ion channel may either protect cells from early tumorigenesis as reported for colorectal carcinoma, an effect mediated by IL-18 (43), or enhance tumor progression favoring invasion and metastasis mainly through an IL-1β-mediated effect (Fig. 1) (44). As carefully reviewed elsewhere (45), findings regarding inflammasome roles in tumor progression are still controversial and highly depend on the tumor type and experimental settings.

Responses to microenvironmental cues may also alter TAMs' purinergic receptor expression patterns. Particular patterns of P2X receptor expression were found in macrophages derived from a mouse model of leukemia. Except for the P2X5 subtype, all P2X receptors were expressed. Upregulation of P2X7 receptor expression matched with disease progress, while P2X1 receptor expression was increased

in spleen-derived macrophages (46). The functions exerted by these upregulated purinergic receptors are still an issue of debate.

P2X7 receptor-mediated immune modulation of TME was reported by De Marchi and colleagues (2019). They observed distinct antitumoral responses in immune cells, when P2X7 receptors had been pharmacologically blocked with the antagonist A740003 compared to P2X7 knockout mice. P2X7 receptor inhibition led to increased IFN-γ levels and also reduced expression of ectonucleotidases, such as cluster of differentiation (CD)39 and CD73, maintaining adenosine levels under control (47), which likely favors a more effective immune response against the tumor (48, 49). In contrast, P2X7 receptor knockout mice showed decreased numbers of CD8$^+$ T cells and augmented numbers of Treg cells. Besides, receptor knockout lead to the overexpression of CD73 ectonucleotidase, stimulating conversion of AMP into adenosine (47).

Immunogenic cell death (ICD) triggered by chemotherapy is an important component of anti-cancer therapy (50). eATP is a main inducer of ICD, together with pre-apoptotic calreticulin exposure and postmortem release of HMGB1 (51). ATP released from dying cells activates P2X7 receptors in dendritic cells (DC), inducing their maturation and migration. These mature DC present dying cell antigens to prime IFN-γ-producing CD8$^+$ T lymphocytes. Accordingly, chemotherapy is ineffective when administered in P2X7$^{-/-}$, as well as in NLRP3$^{-/-}$ or caspase 1$^{-/-}$ mice (51), confirming the importance of ATP release and P2X7 receptor-mediated activities in ICD. Importantly, inhibition of eATP release or P2X7 receptor activity alone is not fully competent in preventing ICD, pointing to a potentiation effect (50, 52).

Recent evidence demonstrates that P2X4 receptors interact with C-termini of P2X7 receptors modulating inflammatory responses and cell death (53). Moreover, although functional P2X4 receptor expression in resting macrophages may be low, it increases upon phagocytic stimuli. However, upon sustained classical activation, P2X4 receptor expression and functionality decrease (54).

Knockdown of P2X4 receptor expression in RAW264.7 macrophages suppressed P2X7 receptor-mediated events, such as IL-1β and HMGB1 release, ROS production, autophagy and ATP-triggered cell death (55, 56). Furthermore, P2X4 receptor activity is required for inflammasome and caspase-1 activation in neurons after spinal cord injury, events usually attributed to the P2X7 receptor (57). In breast cancer cells, P2X4/P2X7 receptor-gated Pannexin-1 channel sensitivity to ATP was induced by Ivermectin, an allosteric modulator of the P2X4 receptor, inducing both apoptotic cell death and a non-apoptotic, inflammatory type of cell death. In addition, further release of ATP and HMGB1 occurs (58) increasing immunogenic responses in the tumor.

Altogether, these findings, besides from demonstrating the deep involvement of purinergic signaling in antitumor immune response and in tumor cell survival itself, raise several questions that are still far from being answered. eATP is an inflammation enhancer, dependent on purinergic receptors acting through recruitment of immune cells and direct release of inflammatory molecules. P2X7 receptors participate in host antitumor responses and in triggering of tumor cell death. However, the pharmacological blockade of this receptor leads to responses diverse from those observed, when host cells have been knocked out for P2X7 receptor expression, an observation probably attributable to the inhibition of these receptors on tumor cells. These findings reinforce the importance of resolving the effects triggered by each receptor when expressed in diverse cell types present in the TME. Furthermore, it is crucial to elucidate whether P2X7 receptor-triggered inflammatory responses are dependent on P2X4 receptor, and if so, in which circumstances. Finally, the upregulation of P2X1 receptors in spleen macrophages of a murine model of leukemia demonstrated that there are vastly unexplored fields of purinergic signaling worth investigating.

Immunomodulation and Tumor Progression

Recruited circulating monocytes join the resident macrophage population of a transformed tissue. Local colony-stimulating factor 1 (CSF-1), chemokines, such as CCL2 and CCL5, and the oxygen gradient in the tumor mass result in the expression of endothelial cell adhesion molecules and promote macrophage chemoattraction and migration (59). There are two subsets of monocytes with opposite roles: classical monocytes, related to pro-inflammatory responses, and nonclassical monocytes, mostly anti-inflammatory. The recruitment of monocytes depends on chemokine release by the tissue and on chemokine receptor expression by circulating monocytes (59). Adenosine acting through A$_1$, A$_{2A}$ and A$_3$ receptors also increases tumor monocyte infiltration (60).

After tumor establishment, resident or monocyte-derived macrophages may undergo M1 to M2 transition (24, 61), creating an immunosuppressive environment that supports tumor growth. Through M2 activation, macrophages acquire a particular gene expression program that reduces cytotoxicity and favors extracellular matrix (ECM) deposition, effects that are intensified by adenosine stimulation, mainly through A$_{2B}$ receptor (62). Therefore, these macrophages have a reduced antigen-presenting ability, supporting cell survival and tissue repair (63, 64). Finally, they help cancer cells evade immune response, giving rise to one of the cancer hallmarks (25, 26).

eATP is sequentially degraded, generating adenosine and ultimately inosine and uric acid through the action of several enzymes. CD39 and CD73 ectonucleotidases are highly expressed in many tumors (48) both in tumor and stromal cells, and are upregulated during hypoxia, favoring extracellular adenosine generation (65). Not only ectonucleotidases are upregulated in their expression rates in anti-inflammatory or hypoxic environments; in fact, both hypoxia and adenosine levels upregulate heme-oxygenase 1 (HO-1) expression (66, 67), a well-known anti-inflammatory enzyme, reinforcing immune suppression. Ectonucleotidases and HO-1 have cytoprotective roles downmodulating inflammatory functions of innate immunity cells (67), and as such, their roles may be protective for tumor cells as well. HO-1 expression levels are downregulated by ATP (66), thus, it is reasonable to hypothesize that ATP might inhibit anti-inflammatory stimuli by

diminishing HO-1 levels and trigger inflammation directly through actions mediated by purinergic receptors.

CD38 is an ectoenzyme expressed in several immune and non-immune cells and generates adenosine through bypassing the sequential dephosphorylation of ATP by CD39 and CD73 (68). CD38 expression in macrophages apparently does not generate an immunosuppressive environment. In fact, CD38 is highly expressed by inflammatory macrophages, stimulated by LPS *in vitro* or in inflammatory and infectious sites *in vivo* (69). Moreover, in patients with hepatocellular carcinoma, high levels of CD38 in the tumor colocalized with CD68, a macrophage marker, correlated to a better prognosis (70), suggesting antitumor immune response. Currently, CD38 is considered a marker of M1-like polarization, as shown in Table 1.

P1 receptors expressed by immune or tumoral cells respond to adenosine in the TME. Indeed, possible outcomes depend on the involved receptor, ranging from growth stimulation to inhibition (48). Adenosine mediates monocyte differentiation into macrophages and multinucleated giant cell formation through A$_1$ receptor activation, which is prevented by A$_{2A}$ receptor activity (63, 71). On the other hand, A$_{2A}$ receptor-mediated adenosine signaling has a broad effect reversing M1 activation, while A$_2$B receptor enhances M2 polarization through IL-4 and IL-13 actions (62, 63).

While preventing M1 activation, A$_{2A}$ receptor-mediated signaling also reduces pro-inflammatory cytokine expression, such as TNF-α (72) and IL-12 (65, 72), and cytotoxic reactive nitrogen/oxygen species like NO. Moreover, adenosine stimulation increases IL-10 secretion (63). These functions displayed by adenosine for macrophage polarization corroborate the actions of adenosine as an anti-inflammatory molecule, being physiologically important to prevent excessive inflammatory responses and to promote wound healing and tissue repair. Nevertheless in TME, adenosine signaling may be related to cancer progression. In mice, inhibition of both A$_{2A}$ receptors and ectonucleotidases was a promising approach, enhancing antitumor immune responses (65).

ATP-mediated P2X7 receptor signaling has been implicated in resolving inflammation in M2 macrophages due to the release of the major histocompatibility complex (MHC) receptor, CD14 and annexins A1, A2, and A4 (31), suggesting that the P2X7 receptor-related secretome may be immunosuppressive after M2 polarization in established tumors. When expressed in cells other than macrophages, as myeloid-derived suppressor cells and cancer cells, the P2X7 receptor may also exert immunomodulatory roles (73).

A$_{2A}$ and P2X7 receptors are both involved in cytokine release by macrophages. When exposed to glioma-conditioned medium, which contains micromolar concentrations of ATP and its metabolites, ATP and AMP hydrolysis was decreased while IL-10 and CCL2 secretion was increased. These observations were consistent with M2-like polarization. In addition, treatment with ATP enhanced IL-6 secretion, which was prevented by a P2X7 antagonist (23). These alterations may contribute to glioma immune escape (23). Despite the dual functions described for the P2X7 receptor, it is

possible to say that in a general way a balance between eATP and adenosine actions shifts macrophage responses toward immune-stimulating or -suppressing roles, respectively (74).

Although well-known that adenosine-triggered responses are mostly immune suppressive, any further details are largely unknown. Whether there is a specific TME context in which P1 receptors are upregulated, or which signaling pathways specifically are triggered, or adenosine-mediated effects directly on cancer cells, all of these points are still poorly explored. Beyond expression upregulation or downregulation of ectonucleotidases, adenosine signaling in the tumor microenvironment deserves further attention with new perspectives.

Metastatic Spread and Macrophage Function

Metastasis is the spread of cancer cells from a primary to a secondary site, in a highly organized way (75, 76). It is a multisequential process consisting of cancer cells invasion into blood or lymphatic vessels, adhesion to vessel walls and extravasation to a new tissue, establishment of a premetastatic niche and subsequent growth (77, 78). Primary tumors influence the metastatic outcome by either secreting specific factors or recruiting myeloid-derived cells for distant sites (17, 79), called premetastatic niches. Macrophages and endothelial cells prepare these niches for cancer cell adherence and growth and monocyte recruitment and polarization (80).

Metastasis-associated macrophages (MAMs) are a subpopulation of macrophages firstly reported in a breast cancer model in 2009 (80), expressing vascular endothelial growth factor receptor 1 (VEGFR1) and CCL2 receptor (CCR2), but neither C–X–C chemokine receptor 4 (CXCR4) nor Tie2 (17), as shown in Table 1. The CCL2/CCR2 axis is important for the establishment of a specific pro-metastatic phenotype, driving the production of CCL3 to activate CCR1 by an autocrine loop, resulting in MAMs accumulation and retention at premetastatic niches (81). CCL2/CCR2 blockade interrupts metastasis *in vivo* (82) and reduces tumor growth in breast, lung, liver, prostate, and melanoma cancer models (83). CCL2/CCR2 signaling has also been related to macrophage polarization, since its blockade increased M1-related gene expression and cytokine production, while decreasing M2-related gene expression (84). Through CCL2 stimulation, head and neck squamous cell carcinoma provoked M2-macrophage polarization with epidermal growth factor (EGF) release, forming a feedback loop that increased cancer cells motility as well as invasion and metastasis (85).

A constitutive release of CCL2 is driven through ATP/uridine triphosphate (UTP)-mediated P2Y2 receptor activity in rat alveolar and peritoneal macrophages (81), suggesting a possible role of this receptor in the acquisition of pro-metastatic properties. In breast cancer cells, activation of P2Y2 receptors, besides upregulating the secretion of molecules involved in premetastatic niche preparation, correlated to a broader infiltration of monocytes both in the primary tumor and in the lung, suggesting a role of this receptor in recruiting monocytes to help tumor progression and metastasis (86).

TNF-α released from M1 macrophages increased metastasis of ovarian cancer cells (13). Accordingly, coculture of breast cancer cells with macrophages increased the invasiveness of cancer cells due to a TNF-α-dependent upregulation of metalloproteinases, including matrix metallopeptidase (MMP)-9 (87). P2X7 receptors are involved in increased enzyme activity of TACE in macrophages (88) leading to elevated TNF-α levels (37), and are directly implicated in MMP-9 secretion (89). Moreover, NLRP3 inflammasome expression and assembly in TAMs, a function described for M1 macrophage P2X7 receptors (31), correlate with tumor cell metastatic abilities (90).

These findings indicate that eATP participates in metastasis promotion through P2Y2 and P2X7 receptors (Fig. 1), which is corroborated by other recent findings (91). A deeper review of specific metastatic steps will follow below.

Tumor cell invasion

Invasion is the first step of the metastatic process. It consists of alterations of tumor cell–cell or ECM adhesion, proteolytic degradation and motility to physically boost a tumor cell throughout the tissue (92).

Wnt/β-catenin signaling is largely implicated in cancer progression. TAMs stimulated by colon or colorectal cancer cells secrete IL-1β, blocking β-catenin phosphorylation and driving its accumulation, finally culminating in transcription activation of Wnt-related genes (93, 94). These genes are implicated in tumor proliferation, invasion and metastasis, and also in the acquisition of stem-cell-like characters (95, 97).

eATP supports breast cancer cell invasion via the P2Y2 receptor/β-catenin axis increasing the expression levels of β-catenin and subsequently of CD44, c-Myc, and Cyclin D1. Pharmacological inhibition of β-catenin/T-cell factor complex by iCRT14 reversed those effects. Accordingly, the P2Y2 receptor is highly expressed in cancer tissues and positively correlated to metastasis, as its expression knockdown reduced both *in vitro* and *in vivo* events (98).

In breast tumors, TAMs express COX-2 both highly and ectopically, which is related to poor prognosis. In this context, COX-2 was essential for maintaining M2 polarization, favoring immune evasion (99). In hepatocellular carcinoma cells, COX-2 overexpression significantly increases migratory and invasive potentials, effects that can be prevented by COX-2 inhibition by celecoxib (100). In fact, COX-2 and β-catenin are particularly upregulated in the invasive front of colorectal tumors, and evidence points to a role of Prostaglandin E2 (PGE2), an enzymatic product of COX-2, in increasing β-catenin accumulation (101). P2 receptors are able to increase COX-2 expression and PGE2 production in human alveolar epithelial carcinoma cells (102). Taken together, these findings allow hypothesizing that (I) COX-2 either from macrophages or from tumor cells may have effects in macrophage polarization and invasion properties and (II) P2 receptors may have a role in any of these pro-invasive processes.

Macrophages also regulate composition and structure of the ECM by either depositing ECM components, such as

collagens, or breaking these components down through MMPs, serine proteases and cathepsins release (103–105). When pancreatic cancer cells were cocultured with macrophages, their invasiveness augmented, as well as the expression of MMP-9 and A disintegrin and metalloprotease domain 8 (ADAM8), key enzymes in tissue remodeling (106). Besides, M2-conditioned medium stimulated SW480 colon cancer cell invasion via increased MMP-9 expression and activity (107). In a murine pancreatic neuroendocrine tumor model, Cathepsin Z, predominantly provided by TAMs, was pointed out as the compensatory protease regulating acquired tumor-promoting functions of neoplastic lesions of Cathepsins B and S-deficient mice (108). P2X7 receptors participate in remodeling of extracellular matrix and in promoting invasiveness of cancer cells by utilizing cysteine cathepsins (109) or metalloproteases, as evidenced by the silencing of P2X7 receptor expression leading to increased activity of MMP-2 and decreased MMP-9 activity (110). Whether P2X7 receptors expressed in macrophages have a role in mediating the release of ECM-digesting enzymes has not yet been investigated.

Up to now, very little is known about purinergic involvement in TAM-mediated invasiveness of cancer cells, and only two receptors have been explored: P2Y2 and P2X7 receptors. Filling this gap might point some directions for treating and preventing metastatic disease, the main cause of death among cancer patients.

Tumor cell extravasation

Extravasation is the process, by which cancer cells attach to blood vessel walls and invade the target tissue (111). Monocytes/macrophages are essential metastasis promoters acting either by preparing sites or regulating extravasation, survival and growth of metastatic cells (80, 112). An example is the increased extravasation mediated by CCR2+ monocytes recruited through metastatic tumor cells and target-site tissue CCL2 release (82). These inflammatory monocytes secrete molecules, such as VEGF-A, increasing vascular permeability and favoring tumor cell extravasation. Besides increasing recruited-monocyte population, CCL2 stimulates the production of CCL3 by MAMs, retaining them at the metastatic site (80–82).

The treatment of metastatic Ewing sarcoma in animal models with a selective inhibitor of M2 function reduced tumor burden, invasive metastasis and extravasation (113), eliciting the involvement of M2 polarization in these pro-metastatic phenomena. Furthermore, CD11b+ MAMs were required for metastatic extravasation, survival and growth. These cells recognize, cooperate and assist extravasation of tumor cells in order to invade the lung parenchyma. Depleting these macrophages, extravasation was inefficient, and tumor cells entered apoptosis, resulting in poor seeding efficiency (80).

Paradoxically, recent data from a three-dimensional (3D) vascularized microfluidic model demonstrated that circulating monocytes reduced tumor cell extravasation in a noncontact manner, while after leaving the blood stream and

reaching the tissue, monocytes became macrophage-like and no longer displayed this activity (114). These opposing findings regarding a pro-extravasation or anti-extravasation activity of macrophages can be conciliated considering that circulating monocytes and differentiated polarized macrophages in the tissue can have distinct roles. Therefore, different types and developmental stages of tumors might partly explain controversial evidence.

Nucleotides, such as ATP, diffuse toward endothelial cell surface and activate P2Y2 receptor, opening endothelial cell barrier and facilitating tumor cell extravasation (115). Genetic deletion of P2Y2 receptor in mice resulted in reduced metastatic spreading, also suggesting a possible involvement in the formation of platelet covered tumor emboli, a process that facilitates cancer cell transport in the bloodstream (48, 116, 117). Moreover, eATP promotes intravascular survival of tumor cells by inhibiting cell deformation-triggered apoptosis through P2Y receptors (118).

To extravasate and consequently metastasize, factors such as transforming growth factor beta (TGF-β) and VEGF (117) act locally by reducing endothelial barrier function, while some adhesion proteins expressed on neutrophils, such as Angiopoietin-like 4, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), and fibronectin on endothelial cells, increase the adhesion ability of circulating cancer cells (119). P2Y2 receptors also mediate expression induction of adhesion molecules. Highly metastatic breast cancer cells release great levels of purines and present high P2Y2 receptor activity, correlated to increased proliferation, elevated expression levels of metalloproteinases and VEGF, and augmented VE-cadherin phosphorylation in endothelial cells (120). Macrophages are recruited less when endothelial activation is blocked by either an anti-VCAM-1 antibody or a Vascular adhesion protein-1 (VAP-1) inhibitor, resulting in reduced metastatic cell survival (121).

Considering the involvement of M2 macrophage phenotype in the metastatic cascade, all of the purinergic pathways previously discussed that either contribute to or prevent M2 polarization could constitute possible therapeutic targets. The P2Y2 receptor, discussed in most sections up to now, is for sure a strong candidate for further investigations.

Purinergic Signaling and Macrophage-Provoked Effects on Epithelial–Mesenchymal Transition

Inflammation is a potent inducer of epithelial–mesenchymal transition (EMT) in tumors (122). Several correlations have been established between macrophage polarization and EMT induction, or between TAM density/spatial clustering and EMT features such as E-cadherin loss, β-catenin accumulation and fibronectin expression (122). TAM density was positively correlated to the number of cancer stem-cell-like epithelial cell adhesion molecule (EpCAM)$^+$ cells and colocalized at marginal sites of hepatocellular carcinoma tumor samples. Also, Hepa1-6 cells exposed to TAM-conditioned medium acquired stem-cell-like and EMT traces, which were blocked by depletion of TGF-β, an important EMT-inducer (123).

Coculturing THP1-derived macrophages with lung cancer cells augmented the release of IL-6, PGE2, and increased expression of COX-2, translocating β-catenin to the nucleus and inducing EMT (124). In contrast to the case of epithelial-like cancer cells, the conditioned medium of mesenchymal-like breast cancer cells induced TAM-like alterations on macrophages through increased secretion of granulocyte macrophage CSF and expression of immunosuppressive-related molecules (125). Secretion of CCL18 by TAMs induced EMT and favored a mesenchymal phenotype, generating a positive feedback loop. In breast carcinomas, EMT increased immunosuppression, protection from immune response and cancer cell survival, linking mesenchymal cancer cell phenotype with M2 polarization (126).

Purinergic receptors are also implicated in EMT. In breast cancer cells, EMT-like changes were induced by either EGF or hypoxia modulated Ca^{2+} signaling through purine receptors (127, 128). In agreement, phenotypic transition significantly changed P2X and P2Y receptors expression profile (128). In distinct cancer types, P2Y2 receptor activation by either ATP or UTP leads to increased expression of EMT-related genes through EGF receptor (EGFR) transactivation, extracellular signal-regulated kinase (ERK) phosphorylation or IL-8 induction (129–134).

P2X7 receptors, besides being implicated in increased VEGF expression, a well-known EMT inducer (135), participate in other signaling pathways converging into EMT induction, as ERK1/2 phosphorylation, phosphoinositide 3-kinase (PI3K)/Akt pathway and release of MMPs, as described in previous topics. MMPs are able to enhance EMT by either processing pro-TGF-β or by direct proteolysis of E-cadherin. In renal tubular epithelial cells, P2X7 receptor alone was able to induce EMT similarly to TGF-β (136). In addition, P2X7 receptor is highly expressed in M2 macrophages and has a different secretome related to this phenotype (31, 131), raising questions about the ability of P2X7 receptor expressed in macrophages to induce EMT as well.

The adenosine diphosphate (ADP)-activated P2Y12 receptor expressed by platelets induced EMT and invasiveness in lung cancer cells through TGF-β release (137). The P2Y12 receptor was also identified on mouse peritoneal resident macrophages (138, 139) and is upregulated under M2 activation conditions on resident human microglia (140), correlating its expression with an anti-inflammatory milieu. P2X4 receptors mediate the release of CXCL5 by macrophages (141), a chemokine involved in EMT and chemoresistance through CXCR2 activation (142). While the P2Y1 receptor exacerbated TGF-β EMT-promoting effects in renal tubular epithelial cells, P2Y11 receptor displayed dual effects upon inhibition of protein kinase A (PKA) or mitogen-activated protein kinase (MAPK) downstream pathways (136). Besides being upregulated in cells stimulated by EGF or hypoxia (128), P2Y6 receptor activation with uridine diphosphate in breast cancer cells increased MMP-9 secretion and activity, and its inhibition or silencing prevented these changes (143).

Stimulation of A$_{2A}$ receptors reduced expression level of EMT-relates genes in renal tubular epithelial cells, while A$_1$

receptors potentialized TGF-β-mediated effects (136). On the other hand, A_{2B} receptor stimulation by itself upregulated mesenchymal gene expression in human epithelial lung cells, while it counteracted EMT-inducing effects when in combination with TGF-β, modulating two downstream pathways with opposing effects (Fig. 1). Thus, A_{2B} receptor arises as a fine regulator of phenotypic transition (144).

Macrophages are able to modulate cancer cells phenotype through the purinergic system by modulating ectonucleotidases, nucleotide transporters or cancer cell receptors that ultimately induce or prevent EMT. Reversely, cancer cells that underwent EMT may modulate immune response and tumor progress throughout nucleotide release, transport or degradation. TGF-β increases CD73 expression (145), favoring adenosine generation and anti-inflammatory stimuli. Moreover, adenosine accumulates in the TME after apyrase activation favoring an epithelial-like phenotype (131).

The purinergic system, though, is still highly unexplored regarding its effects on macrophage-mediated EMT. Several lines of evidence mainly consist of connections between macrophage polarization, EMT and purinergic signaling in pairs, but experiments actually evaluating the interaction between the three factors are scarce and would be valuable. Besides adenosine receptors, evidence now points to P2Y2 and P2X7 receptors. Additionaly, evidences also raise many possibilities regarding other purinergic receptors, as the P2Y12 receptor that may be involved in TGF-β secretion and EMT induction, which for sure deserves further investigation. Once more, the oversimplification of correlating eATP to inflammatory and antitumor activities proves itself too trivial to be accurate.

Purinergic Signaling in TAM-Mediated Angiogenesis

Blood vessels are originated or enriched by two ways: vasculogenesis and angiogenesis (146). Vasculogenesis comprises the transformation of angioblasts into endothelial cells and then vessels during embryonic development. Angiogenesis originates blood vessels from pre-existing ones by sprouting or by bone marrow stem cells both during embryonic development and adult vascular remodeling. Therefore, during i.e. pregnancy and wound healing, angiogenesis supports tissue homeostasis by specific molecular signaling pathways involving HIF-1α, VEGF, basic fibroblast growth factor (b-FGF), platelet-derived growth factor (PDGF), and Angiopoietin-1 (147–153).

Unfortunately, these pathways can also be triggered and misused by pathological conditions (148). In tumors, angiogenesis is activated to provide nutritional supply in order to achieve large-scale growth. Usually, hypoxia and nutritional deprivation develop, once the tumor has reached some millimeters, leading to cytokine and growth factor release, such as VEGF, inducing quiescent endothelial cells to proliferate and sprout. Since tumor angiogenesis is not well coordinated, there is leakage of platelets and increased levels of PDGF, which recruits perivascular cells (154). In addition, tumor-associated fibroblasts deposit high amounts of ECM components, which can be cleaved by MMPs leading to epitope exposition and inflammatory cells recruitment (154).

Firstly, the inflammatory milieu attracts circulating monocytes that differentiate into mature TAMs. Moreover, associated endothelial cells secrete Angiopoietin-2, recruiting Tie2-monocytes that escalate the angiogenic response (154–156). The hypoxic TME presents not only pro-inflammatory molecules, but also higher levels of adenosine and eATP. Adenosine-producing enzymes such as CD39 and CD73 are overexpressed under Sp1-mediated HIF-1α stimulation (157). On the contrary, deletion of these enzymes improves antitumor immunity and survival (158, 159). In a mouse model of breast cancer, blockade of CD73 impaired its pro-angiogenic effects mediated by enzymatic and non-enzymatic pathways (160). In fact, adenosine is a master regulator of angiogenesis, and all P1 receptors have roles in modulating angiogenic factors. A_1 and A_{2B} receptors upregulate IL-8, while A_{2A} receptors stimulate IL-10 release. VEGF levels are increased by A_{2A}, A_{2B}, and A_3 receptor activities; HIF-1α is elevated in response to A_3 receptor stimulation, and iNOS/endothelial NO synthase (eNOS) expression levels are upregulated, respectively, as consequence of A_{2A} and A_{2B} receptor activities (161). Interactions between murine A_{2A} and TLR-2, -4, -7, and -9 in macrophages have been reported, upregulating VEGF and downregulating TNF-α generating a so-called angiogenic switch (162).

P2Y receptors may promote endothelial cell tubulogenesis mediated by tumor-secreted nucleoside diphosphate kinase (NPDK). In addition, P2Y1 receptor activation leads to VEGF receptor 2 (VEGFR-2) transactivation, independently from VEGF, increasing the angiogenic response (Fig. 1) (163). Similarly, the P2X7 receptor induces angiogenesis by modulating the PI3K/Glycogen synthase kinase 3 beta (GSK3-β)/VEGF pathway (164).

Despite evidence highlighting P2X7, P2Y1, and P1 receptor roles in angiogenesis, the involvement of macrophages and monocytes is poorly investigated. However, it is likely that the purinergic system, highly present and active in macrophages, mediates effects toward pro- and antiangiogenic responses.

CYTOMETRY IN MACROPHAGE POLARIZATION ANALYSIS

Comprehension of macrophage phenotypic changes and its diverse functions is clearly fundamental for understanding how macrophages affect the TME. Therefore, the polarization footprint of these cells within tumors may work as a prognostic indicator and improve decision making regarding therapies. Experimentally assessing macrophage polarization nowadays relies on characterizing enzymatic activity, surface markers expression, specific genetic programs and morphology (10). Enzymatic activity is commonly assessed measuring nitrite dosage and arginase activity enabling classification of M1 and M2 murine macrophages, since M1 types present higher NO levels, while M2 macrophages display higher arginase activity (10, 165). The resolution between intermediate phenotypes and the corroboration of initial observations should thus rely on high-resolution methods able to quantify transcription products and

protein levels, as reverse-transcription quantitative polymerase chain reaction and imaging or flow cytometry (17, 165).

Flow cytometry analysis has turned to be a crucial technique for determining macrophage activation and immune status. For instance, macrophage phenotype analysis of a mouse model of colon carcinogenesis was done by immunostaining against CD14, CD3, CD68, CD204, and CD206 followed by flow cytometry analysis (166). The presence of the anticancer drug triptolide inhibited differentiation into tumor-associated M2 macrophages as shown by decreased expression of M2 phenotypes.

Further phenotype changes of macrophages in contact to tumors were elucidated by flow cytometry. Tumor-associated macrophages expressed CD163, CD204, and CD206. Biopsy samples of these cells dissociated and submitted to flow cytometry analysis revealed that CD206$^+$ macrophages produced more EGF when compared to CD163$^+$ and CD204$^+$ macrophages (167). EGF production by CD206$^+$ macrophages were correlated with bad clinical prognosis.

CD38 expressed on macrophage surfaces are involved in immune suppression mechanisms in cancer. Coexpression of CD38 together with the macrophage marker CD68 was measured in tissue samples from patients with hepatocellular carcinoma for establishing a prognosis based on CD38^{hi} macrophage density (70).

Flow cytometry together with microscopy techniques may enrich the observations with marker localization and cell morphology in culture, in histological tumor slices from patients, or even xenograft models. Cancer cells are engulfed by macrophages, being important for innate cancer immunity. Nam and coworkers reported an assay for phagocytosis based on flow and image cytometry (168). The CellTracker dye used for flow cytometry measured the phagocytosis of 4T1-Luc, B16F10-Ova, CT26.CL25, and HT29 cancer cells, while microscopic analyses with the pH-sensitive dye (pHrodo) allowed to visualize tumor cells surrounded by macrophages.

Quantitative immunofluorescence assays have been used for studying macrophage presence and phenotypes. For instance, lymphnode biopsies were studied for the quantification of the ratio between CD30$^+$ and CD68$^+$ lymphoma-associated macrophages and Hodgkin–Reed–Stemberg cells (169). Quantitative imaging of immunostained macrophages and microglia obtained from mouse IPS cells showed that these were capable of recapitulating pro- and anti-inflammatory pathways in the brain (170).

Above-mentioned examples illustrate that quantitative information can be obtained by imaging cytometry, an approach that combines visual information with precise cytometric quantification (171–173).

Cytometry becomes an even more powerful tool when using nanobodies. Consisting of single variable domains derived from heavy chain camelid antibodies, nanobodies provide in addition to high specificity, wider penetration in live tissues (174–175), the ability to distinguish between protein conformational states, and even to modulate enzymatic activity of a cellular target through interactions with the binding epitope. Fumey and coworkers took advantage of nanobodies' easier tissue penetration to perform in vivo live imaging with fluorescently labeled anti-CD38 nanobodies (175). In fact, this approach allows studying macrophage phenotype inside a living tumor or even modulating CD38 enzymatic activity to control immune response (175, 176). Coupled with image cytometry analysis techniques, quantitative information extracted from such sources might be disruptive.

A clear limitation of measuring adherent cells by flow cytometry is the required detachment from their 3D tissue context, altering morphology and disrupting all cell–cell interactions. Preparation for flow cytometric measurements, such as enzymatic digestion of adhesion matrix or mechanical disruption, destroys cell surface epitopes and affects the cellular phenotype. Slide-based or tissue cytometry equipment provides automation of microscopy scanning and suitable software for image analysis. Images can be acquired in bright field or fluorescence mode, allowing simultaneous quantification of genes relative expression and protein markers, morphologic features, and cellular or subcellular localization, among other parameters.

Fluorescence-based flow cytometry, despite several advantages, has a limited resolution while detecting multiple targets due to the spectral spillover of fluorochromes (177). An important alternative that improves resolution is mass cytometry, also known as time-of-flight cytometry (CyTOF). In CyTOF, the antibodies bound to cellular targets are labeled with highly purified metal isotopes, which are then measured in a time-of-flight mass spectrometer. Several advantages come with CyTOF, including an easier panel design that allows performing a detailed phenotypic or functional analysis of a cellular sample, all at once and with minimal overlap between channels. The technique allows quantitative measurements of at least twice as much targets as would be feasible with fluorescence (178). Besides samples in suspension, tissue sections may also be analyzed through imaging mass cytometry (179).

Advanced pattern-recognition algorithms have been developed to be used in microscopy-based multicolor tissue cytometry for analysis of spatial distribution of cells and tissue morphology (171). Summing up as an advantage of this technique, the ability to provide high statistical strength with very small-sized experiments highlights imaging cytometry as a very cost-effective method. In a single well of a 96-well culture plate, tens of thousands of single cell events may be quantified. In addition, lamina preparation is unnecessary, since images can be directly obtained from the culture plate. Image cytometry made it easy to measure, for instance, the length of cytoskeleton filaments, or to calculate the number or area of intracellular organelles.

Quantification of canonical markers in nonpolarized (M0), classically activated (M1) and alternatively activated (M2) macrophages is provided as an example of this powerful tool (Figs. 2–4). The whole-well/slide-scanning ability of an image or tissue cytometer allows fast acquisition of high-quality images covering the whole extension of the culture
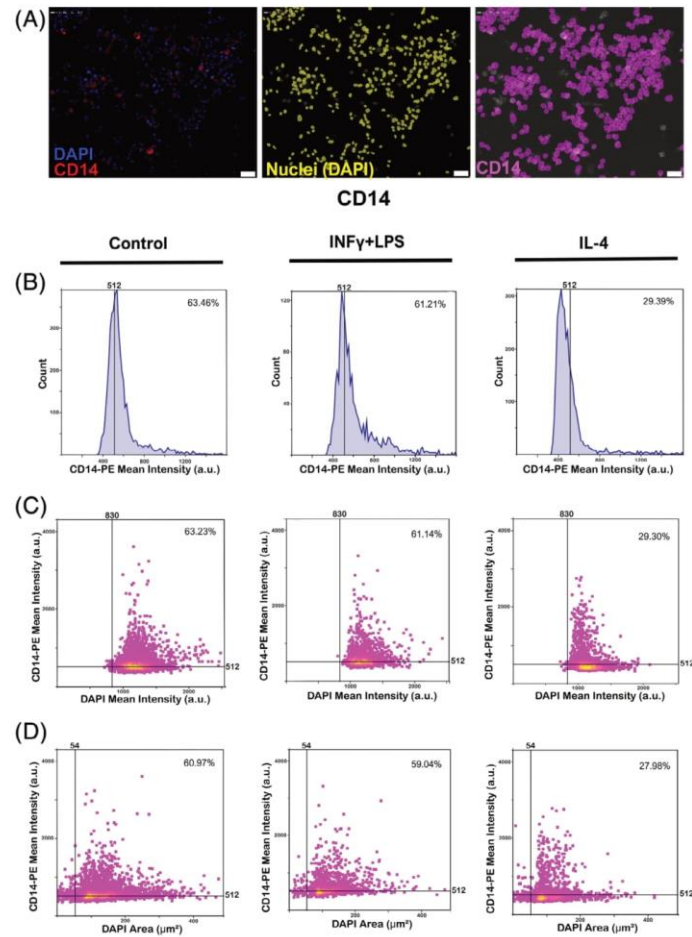
Figure 2. CD14 staining—myeloid lineage marker. (A) Fluorescence microscopy images in a nonpolarized macrophage culture showing DAPI and CD14-PE conjugated antibody costaining. Masks set by the imaging cytometry software StrataQuest (TissueGnostics) recognize cellular areas: nuclear mask (in yellow) recognizes the nuclear area; and cytoplasmic mask (in pink) recognizes the cytoplasmic area. Scale bars represent 50 μm. (B) Histograms showing CD14 mean fluorescence intensity distribution. (C) Dot-plots representing events distributed by DAPI mean intensity versus CD14 mean fluorescence intensity. (D) Dot-plots representing events distributed by DAPI area versus CD14 mean fluorescence intensity. Graphs display results of control, M1-polarized (IFNγ + LPS) and M2-polarized (IL-4) conditions. Image acquisition was done with the TissueFaxs System (TissueGnostics, Vienna, Austria) and further analyzed with the StrataQuest software (TissueGnostics). [Color figure can be viewed at wileyonlinelibrary.com]

well or slide, avoiding the selection of specific regions that could lead to an untruthful quantification. Analysis of small regions of interest results in quantitative data with statistical significance in the assessment of molecular markers in cancer (180).

Macrophages were treated for 48 h with 100 ng/ml LPS and 20 ng/ml IFN-γ for promotion of M1 polarization, while M2 polarization occurred by chronic treatment with 20 ng/ml IL-4. CD14 was immunolabeled as a canonical myeloid lineage marker (Fig. 2); CCR7 expression was monitored for M1
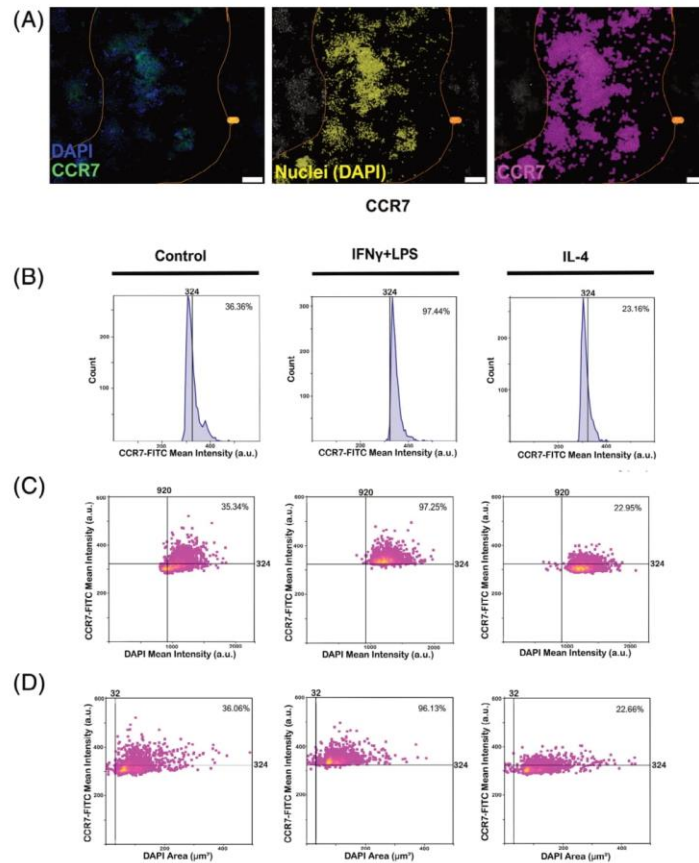
Figure 3. CCR7 staining for M1 phenotype determination. (A) Fluorescence microscopy images in a M1-polarized macrophage culture showing DAPI and CCR7-FITC conjugated antibody costaining. Masks set by the imaging cytometry software StrataQuest (TissueGnostics) recognize cellular areas: nuclear mask (in yellow) recognizes the nuclear area; and cytoplasmic mask (in pink) recognizes the cytoplasmic area. Scale bars represent 200 μm. (B) Histograms showing CCR7 mean fluorescence intensity distribution. (C) Dot-plots representing events distributed by DAPI mean intensity versus CCR7 mean fluorescence intensity. (D) Dot-plots representing events distributed by DAPI area versus CCR7 mean fluorescence intensity. Graphs display results of control, M1-polarized (IFNγ+LPS) and M2-polarized (IL-4) conditions. Image acquisition was done with the TissueFaxs System (TissueGnostics, Vienna, Austria) and further analyzed with the StrataQuest software (TissueGnostics). [Color figure can be viewed at wileyonlinelibrary.com]

phenotype characterization (Fig. 3), while CD23 and CD163 were employed as M2-phenotype markers.

In Figures 2–4A, we show fluorescence images comprised of macrophage cultures costained with 4′,6-diamidino-2-phenylindole (DAPI) and specific antibodies targeting molecular markers, chosen to demonstrate an example of how fluorescence quantification works in an imaging software.

First, the software needs to recognize individual events by setting a mask. In our example, we started by delimiting nuclear masks using DAPI nuclei staining, that recognizes a single nucleus. The nuclear mask is visible in Figures 2–4A, in the middle section, in yellow. For that, the user configures the mean size of the nuclei and the fluorescence background threshold. Based on the nuclear mask, a cytoplasmic mask is set and fluorescence measurements can be obtained by both
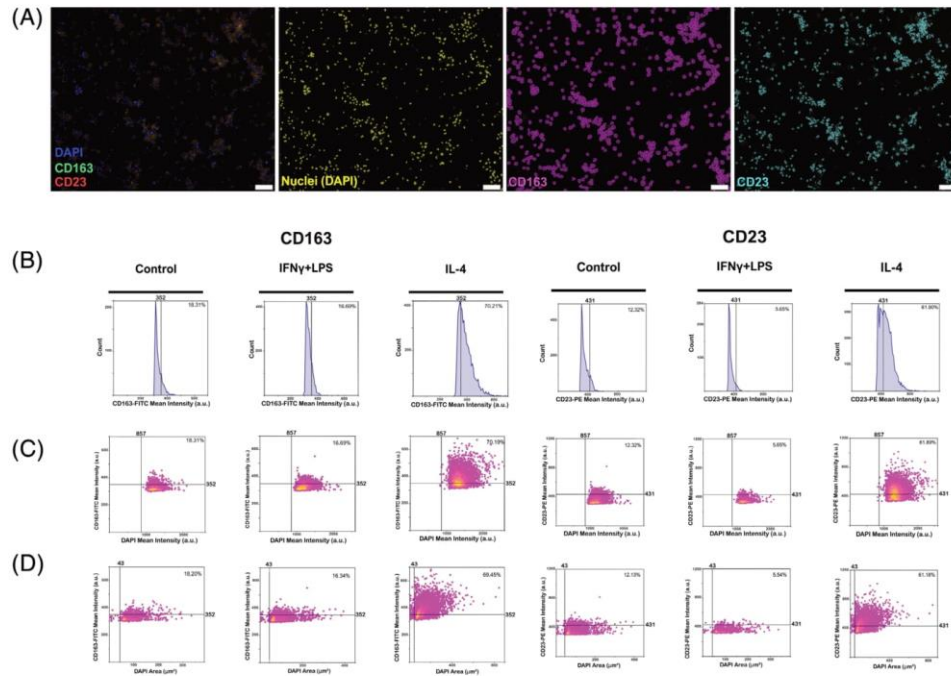
Figure 4. CD163 and CD23 staining—M2 phenotype markers. (A) Fluorescence microscopy images in a M2-polarized macrophage culture sample region showing DAPI and CD163-FITC and CD23-PE conjugated antibodies costaining. Masks set by the imaging cytometry software StrataQuest (TissueGnostics) recognize cellular areas: nuclear mask (in yellow) recognizes the nuclear area; and cytoplasmic masks (in pink and turquois blue for CD163 and CD23, respectively) recognize cytoplasmic area. Scale bars represent 100 μm. (B) Histograms showing CD163 and CD23 mean fluorescence intensity distribution. (C) Dot-plots representing events distributed by DAPI mean intensity versus CD163 or CD23 mean fluorescence intensity. (D) Dot-plots representing events distributed by DAPI area versus CD163 or CD23 mean fluorescence intensity. Graphs display results of control, M1-polarized (IFNγ+LPS) and M2-polarized (IL-4) conditions. Image acquisition was done with the TissueFaxs System (TissueGnostics, Vienna, Austria) and further analyzed with the StrataQuest software (TissueGnostics). [Color figure can be viewed at wileyonlinelibrary.com]

including or subtracting the nuclear area, inside or outside any cellular compartment. Specific masks for cellular structures of interest can also be set, as filaments, vesicles or dots. In our example, also shown in Figures 2–4A, cytoplasmic masks are shown in pink or turquoise blue, as identified in captions.

While whole well scanning increases the statistical power and the reliability of results, the selection of specific regions based on whole-well visualization can be employed to reduce the number of events to be analyzed, speeding up the process, or to discard blurred or poorly confluent regions. In Figure 3A, an orange contour delimiting a specific region of interest selected for fluorescence quantification is visible, also as an example.

By mask segmentation, several parameters, including area, fluorescence mean intensity and sum intensity, as shown in B, C, and D of Figures 2–4, were measured. From these data scatter-plots, dot-plots and histograms were created. The software can automatically detect colocalization of markers, at the single-cell level in a quantitative manner. Finally, gates are set by the user to identify the frequency or count of specific events in a selected population. Quantitative results are summarized in Table 2. As shown here, numerical evaluations of observer-independent data are now available based on a significant number of individual measurements. LPS and IFN-γ-treatment increased the number of CCR7^{+} cells (Fig. 3) and subsequently the pro-inflammatory M1 phenotype. Following IL-4 treatment frequencies of CD163^{+} and CD23^{+} cells augmented (Fig. 4), while at the same time the frequency of CD14^{+} cells diminished (Fig. 2), being consistent with the acquisition of an anti-inflammatory M2 phenotype.

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Table 2. Quantitative of immunostaining obtained by image cytometry

	CONTROL (NONPOLARIZED)	IFNγ + LPS (M1-LIKE)	IL-4 (M2-LIKE)
CD14+ cells	**61%**	**59%**	30%
Total event counts	4,253	1,526	3,277
CCR7+ cells	36%	**96%**	22.7%
Total event counts	1,664	1,600	1,403
CD163+ cells	18%	16%	**69.5%**
CD23+ cells	12%	5%	**61%**
Total event counts	2,589	956	5,649

Percentage data were extracted from the dot-plots represented in Figures 2–4D, in which M1 and M2 markers fluorescence mean intensity was plotted versus DAPI area. Bold values represent the highly positive samples for each marker.

Quantitative determination of immunophenotypically identified populations in a cell culture, xenographic tumor tissue sections and metastatic niches will significantly increase our knowledge and understanding of the interactions between different cell types in the TME. Cells with similar or identical morphologies, but with different molecular signatures and functions can be discriminated. Finally, due to the automated processing, biases depending on the observer are avoided, and results are highly reproducible. Multiplex epitope quantification by tissue cytometry can be performed for further macrophage phenotyping in the tumor neighborhood (181).

FINAL REMARKS

Inflammation and immunomodulation are crucial for determining cancer cell fate, being able to drive them more or less aggressive, prone to invasiveness and metastasis or to cell death. Uncovering the links between these phenomena is fundamental to advance in cancer research field, and extracellular purines are highly present and significant in this context. Here, a comprehensive review highlights some of the many potential targets for purinergic signaling investigation (Fig. 1).

The majority of microscopy-based investigations are currently assessed visually, so as to "look and conclude". Although important gross effects can be identified by a human observer, complex interdependencies between different cellular events are often beyond our ability to observe and remain unknown. Especially in oncology, techniques must allow the quantitative analysis of multiple markers in a high-scale number of individual cells simultaneously, rather than just visual evaluation. Algorithm optimization is challenging and takes time. However, automated machine learning has been successfully used. The challenge lies now in the deep learning engines for image cytometry, such as training the image analysis program in setting optimal values of trainable parameters, for instance for nucleus segmentation of fluorescence images (182, 183).

Assessing the phenotype of macrophages is essential to understand their influences in the tumor and to uncover novel prognostic predictors and target pathways for therapeutic interventions. A continuous and deeper investigation of the purinergic signaling system in the TME employing cut-edge experimental tools may be a key strategy to reach novel possibilities for cancer management.

CONFLICT OF INTERESTS

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Review

Calcium signalling: A common target in neurological disorders and neurogenesis

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HIGHLIGHTS

- Alteration in $[Ca^{2+}]_i$ homeostasis mediates Alzheimer's disease pathophysiology.
- mHtt leads to $[Ca^{2+}]_i$ disbalance upon NMDAR activation.
- L-type VGCC controls pacemaking in Parkinson's disease neurons of the Substancia Nigra.
- Rhythmic Calcium signalling induces neuronal differentiation of NSCs.
- In vitro models of neurological diseases by iPSC corroborate $[Ca^{2+}]_i$ signalling roles in disease onset.

ARTICLE INFO

ABSTRACT

Calcium is an ubiquitous second messenger used by any living cell. The fine-tuning of intracellular free calcium concentration ($[Ca^{2+}]_i$) homeostasis and signalling pathways is crucial for the maintenance of the healthy organism. Many alterations in the homeostasis can be compensated by robust mechanisms; however, cells that already present some debility in those mechanisms, or that are over stimulated cannot compensate the stress and die. Many neurological diseases show $[Ca^{2+}]$ disbalance as trigger of apoptotic response resulting in massive neuronal loss and the neurodegeneration. In this review, we focus on presenting similarities and differences of neurological disorders like Huntington's, Parkinson's, Alzheimer's disease and schizophrenia and the current clinical trial status. Moreover, we describe the importance of Ca^{2+} signalling in neurogenesis, showing that interference of this signalling could go along with stem cell therapy in the central nervous system.

1. Introduction

Intracellular calcium is an universal second messenger [1,2] in mammalian cells ranging from cytosolic Ca^{2+} concentration from 10 to 100 nM at rest. By evolution means, Ca^{2+} ions were chosen as the ubiquitous messenger, probably parallel to ATP emerging as the intracellular energy transfer molecule, because the presence of Ca^{2+} ions during ATP production leads to the formation of insoluble phosphate salts. Since the concentration of Ca^{2+} ions in the cytoplasm is much lower than the environment surrounding the cell and the endoplasmic reticulum, the molarity driven force points to inward direction into the cytosol [3]. There are efficient tool kits based on Ca^{2+} buffers, transporters and pore formation that equilibrate the transmembrane fluxes, scavenge the excess of Ca^{2+} out of the cell or to intracellular membrane compartments [4–15]

Cell toxicity induced by calcium-increased levels in the cytosol is well described for over 30 years, and the toxic levels can lead to damage and cell death [16–18]. For example, acute insults by Ca^{2+} ion overload will trigger necrotic cell death [19], and simultaneously can start the signalling for programmed cell death, called apoptosis, which is a key process in embryo development and in many pathologies [19,20]. Moreover, chronic changes in Ca^{2+} signalling contribute to the pathogenesis of many diseases [3,21–25], including neurodegenerative and psychiatric disorders, such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's diseases (PD) and schizophrenia. All mentioned pathologies impose medical, financial and social problems since they are incurable, encouraging the need to develop novel treatments and approaches. Thus, in this review, we focus on altered intracellular Ca^{2+} signalling and its potential as a therapeutic target for neurological disorders. Moreover, we highlight how calcium signalling participates in the maintenance and differentiation of neural precursor cells for improved cell therapy.

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2. Intracellular calcium signalling in healthy neurons

Intracellular Ca^{2+} signalling in neurons is extremely fine-tuned, because it controls survival, gene transcription, membrane excitability and many other cellular processes [26–29], including synaptic plasticity [30]. Changes in free intracellular calcium concentration ($[Ca^{2+}]_i$) levels trigger downstream signalling pathways targets like kinases, phosphatases, transcription factors, enzymes and proteins that induce synaptic vesicle fusion [27,31,32].

Entrance of Ca^{2+} from extracellular medium in to cytosol of neurons occurs through voltage-gated Ca^{2+} channels (VGCC), N-methyl-D-aspartate receptors (NMDAR), calcium-conducting α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), canonical transient receptor potential (TRPC) channels and calcium release activated channels (CRAC) among others. Efflux of Ca^{2+} from intracellular stores like the endoplasmic reticulum (ER) is controlled by ryanodine (RyR) and 1,4,5-triphosphate receptors (IP3R). Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is activated, when cytosolic Ca^{2+} levels are increased, and transport ions back to the ER lumen [32]. There are some Ca^{2+} sensor proteins, like stromal interacting molecules (STIMs) that present an EF-hand Ca^{2+} binding domain [33] located in the ER lumen, and serve as sensors of intracellular calcium concentration stores [34], that upon depletion oligomerize and translocate to the cytosol, leading to the opening of the plasma membrane channels Orai family [35], encoding Ca^{2+} release-activated Ca^{2+} current (ICRAC), and non-selective cation channels of TRPC family encoding store-operated current (ISOC) [6,35–37]. In this way, there is a replenishment of the stores occurs, the name of this process is store-operated calcium entry (SOCE) [32,38].

Regarding intracellular calcium signalling, there are striking molecular similarities between neurological disorders and important consequence for these diseases, reinforcing this pathway as a great target for future treatments.

3. Intracellular calcium signalling in neurological disorders

3.1. Alzheimer's disease

Alzheimer`s disease (AD) is a neurodegenerative disorder, that compromises the formation and storage of memory. The onset of symptoms occurs at an age of around 60 years old in sporadic cases and earlier on in familial cases [39]. The latter is usually caused by mutations in the genes encoding for Presenilin 1 (PS1) and 2 (PS2), and amyloid-precursor protein (APP) [39–41].

The main hypothesis for AD is the amyloid cascade stating that an increased/disorganized production of amyloidogenic Aβ42 peptide reduce the number of synapses and promote neuronal cell death [32,41,42]. β-secretase and γ-secretase are protease complexes that cleave APP into the toxic Amyloid β (Aβ) peptides, in which presenilins are part of the catalytic site of β-secretase [43]. However, clinical trials trying to eliminate or modify the production of Aβ from the brain failed to benefit patients [44–46].

Recently, a new hypothesis that states $[Ca^{2+}]_i$ homeostasis dysregulation as the cause of the disease is supported by a wide range of studies [21,32,47,48]. Summarizing, the levels of Ca^{2+} in the ER are elevated in both AD and aging neurons [21,32,47,48]. High Ca^{2+} levels lead to a compensatory mechanism that unbalances phosphatase calcineurin (CaN) and calmodulin-dependent protein kinase II (CaMKII) expressions levels. These proteins are intensively located at synapses, and this disbalance shifts long-term potentiation to long-term depression, leading to memory impairment, synaptic loss and neurodegeneration [32,49–51].

In addition, Aβ peptides can elevate the steady state of Ca^{2+} levels in the neurites by increasing calcium influx through L-type VGCCs and NMDA receptor activation [32,52–57]. Excitotoxicity triggered by NMDA receptor over activation was proven by challenging of with Aβ

oligomers *in vitro* cultured cortical neurons, which led to an abrupt rise in $[Ca^{2+}]_i$ levels [32,58–60]. Thus, neuroprotective effects were shown as result of NMDA receptor blockade by memantine, a non-competitive antagonist already being used for AD treatment [61]. Memantine's and its efficacy is probably due to the blockage of the excessively activated receptor activity s [32,56].

Moreover, the brain shows some compensatory mechanisms by endocytosing NMDARs through STEP61 phosphatase [62] or by downregulating GluN1 subunit expression of NMDARs, as observed in post-mortem AD patients' tissues [32,63].

Aβ peptide is not the only molecule with importance for the $[Ca^{2+}]_i$ overload AD hypothesis. Presenilins are also overloading ERs of AD neurons and leading to excessive Ca^{2+} release through IP3R activation [32,64–70]. Mutated presenilin present in AD may form pores at the ER, because they fold together with chloride channels [71] and are large enough to allow passage of small ions [32,72].

Thus, RyRs are also over activated in AD patients, and their short-term inhibition with dantrolene proved to be effective for $[Ca^{2+}]_i$ signal stabilization, cognitive decline amelioration, neuropathology reduction, and decreased amyloid load and memory impairments in AD models [32,73–75].

Beyond the participation of RyRs and NMDAR, the SOCE pathway can also play an important role at in the pathogenesis of AD. Different models of mutant Presenilin, like knock-in PS1- M146V-KI hippocampal mouse samples and AD patient fibroblasts with familial cases or cortical samples from sporadic ones, showed lower expression of the SOCE ER Ca^{2+} sensor STIM2 [76,77]. The lack of STIM2 leads to depletion of ER stores, resulting in reduced mushroom dendritic spines [77,78]. Models of application of Aβ peptides also demonstrated poor synaptic SOCE [32,79,80]. Altogether, treatment with pharmacological activators of SOCE restored mushroom spine deficiency in hippocampal neurons from presenilin and APP-based AD mice models [32,77,79,80] (Fig. 1).

3.2. Huntington's disease

An elongation of the N-terminal CAG trinucleotide stretch of the Huntingtin gene (Htt) causes the autosomal dominant inherited Huntington's disease [81]. Expansions over 35 repeats of glutamine characterize the disease, and longer repeats lead to earlier symptom's onset [82]. Cells primarily affected by mutated Htt (mHtt) are GABAergic medium-sized spiny neurons (MSNs) from the caudate and putamen nuclei in the striatum [32,83], resulting in mood and cognition impairments and disorganized uncontrolled movements.

It is clear that dysregulation of $[Ca^{2+}]_i$ homeostasis plays important roles in HD [84], occurring mainly through NMDAR activation. The mHtt expression leads to accelerated delivery of NMDARs to the plasma membrane, resulting in extra synaptic NDMAR expression in MSNs [85–90]. This shift depends on calpain and STEP phosphatase activation [91] and induces cell death [32,89,90,92]. NMDAR blockage with memantine was neuroprotective both in HD MSN in vitro cultures as well as in HD mice models, in which the drug decreased neuropathological and behaviour symptoms [93,94], such as observed for AD [95].

The mHtt can also bind to other proteins and disbalance other signalling pathways, like VGCCs and IP3Rs [32,96,97]. The binding to IP3R increases affinity for its ligand IP3 and enhances the $[Ca^{2+}]$ levels during response to mGluR1/5 trigger [96,97]. Moreover, mHtt also increases the leakage of Ca^{2+} through RYR activation, leading to store depletion. Thereby, SOCE signalling is triggered [98,99], as shown by increased expression of TRPC and STIM2 in the striatum of YAC128 HD mice [100,101]. RYR, SOCE or STIM2 inhibition results in neuroprotective effects, such as proposed for AD [32,102,103].

3.3. Parkinson's disease

Like HD, Parkinson's disease (PD) is a neurodegenerative disease presenting motor symptoms, as a consequence of the loss of
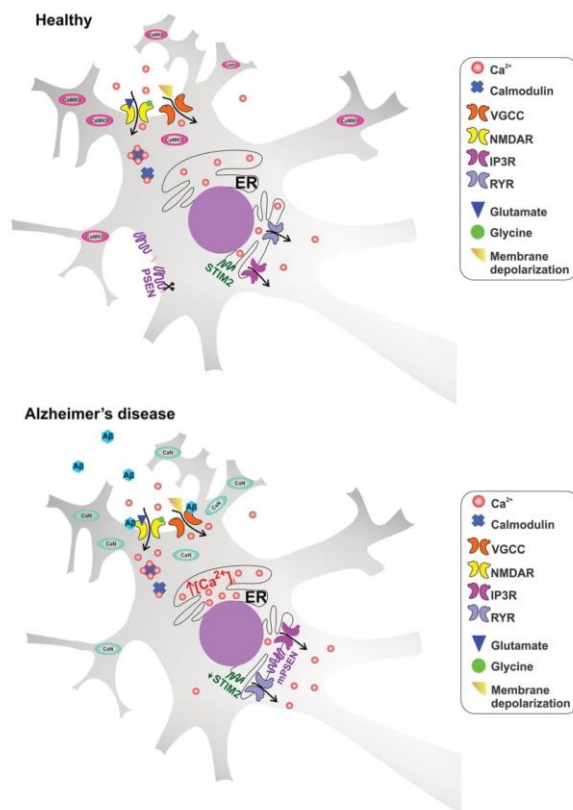
Fig. 1. Ca^{2+} signalling in healthy and Alzheimer's disease patients. In healthy individuals, Ca^{2+} entry is mediated by voltage-gated calcium channels (VGCCs) upon membrane depolarization or by the opening of NMDAR pores following glycine and glutamate binding. In the cytoplasm, Ca^{2+} binds calmodulin to trigger downstream cellular events, and CAMKII is present. In the endoplasmic reticulum (ER), IP3Rs and RyRs drives Ca^{2+} release. Presenilin (PSEN) is localized in plasma membrane. In Alzheimer's disease patients, β-amyloid plaques enhance Ca^{2+} entry through VGCC and NMDAR. A misbalance between phosphatase calcineurin (CaN) and CAMKII expression and activities emerges, leading to downstream alterations in cellular events. Mutated presenilin (mPSEN) is highly localized in the ER membrane, leading to excessive Ca^{2+} release through IP3R and pores, and $[Ca^{2+}]_i$ is thus increased. STIM2 expression is downregulated, impairing SOCE activity.

dopaminergic neurons in the *substancia nigra* [104]. The disease is characterized by the presence of Lewy bodies, which are α-synuclein intraneuronal inclusions, and a decrease in synaptic dopamine secretion [43]. Differently from HD, PD occurs mostly sporadic, and only about 10–15% of the cases are inherited [42]. Genes mutated in PD are called PARKs and mainly affect endo and exocytosis in the synapse, autophagy, endosomal trafficking and mitochondrial housekeeping [105,106].

Most of the genetic related cases are linked to mitochondrial dysfunction as a consequence of a disbalance in $[Ca^{2+}]_i$ signalling [107–109]. During rest, SN dopaminergic neurons show spontaneous Ca^{2+} pace making activity due to influx of Ca^{2+} through L-type VGCC Cav1.3 [110–112]. The constant influx of Ca^{2+} makes mitochondria more susceptible to oxidative stress, that along aging cannot be overcome by compensatory mechanisms [32,112–114].

Some L-type VGCC blockers like isradipine have been studied in PD mice models and demonstrated to be effective in restoring pace making and protecting from death insults [112,115]. In addition, a phase 3 clinical trial for efficiency evaluation of isradipine treatment of PD patients is under way, so far with inconclusive data yet..

Dihydropyridines (DHPs) inhibiting L-type VGCC are already being used as a medicine to treat hypertension and interestingly reduce the risk of PD onset [116–118]. TRPC1 participating in SOCE response

modulates the L-type VGCC in adult SN dopaminergic neurons, leading to decreased frequency and amplitude of pace making activity [119]. Another indication of SOCE in dopaminergic neurons is that a mutation of the Orai1 channel (opened to replenish empty stores) results in decreased tyrosine hydroxylase expression, interfering with production of dopamine [32,120]. In summary, SOCE can play an important role in PD pathology, such as described for AD and HD.

3.4. Schizophrenia

Schizophrenia is a psychiatric disease that like HD has the onset in early adulthood and is linked to genetic alterations. However, there are over 1700 genes related to the disease that cause neurotransmitter dysfunctions such as dopamine-, glutamate- and GABA-mediated transmission [121–123].

Many of the genes related with schizophrenia play important roles in $[Ca^{2+}]_i$ homeostasis whose expressions find altered in central nervous system. For instance, the expression of the regulator of G protein signalling-4 (RGS4) that inhibits the Gq protein, is decreased in the temporal cortex of patients [124,125], while expression levels of the growth-associated protein 43 (GAP 43) that inhibits binding of Ca^{2+} to calmodulin (CaM) is increased in patients' cortex and hippocampus [126,127].

Another category of proteins present in reduced levels in the cortex of schizophrenic patients are the Ca^{2+} buffering proteins parvalbumin, calbindin D28 K and Bcl-2, leading to increased $[Ca^{2+}]_i$ levels and triggering pro-apoptotic responses and neuronal death [127,128]. Moreover, down-regulation of NMDAR gene expression levels are also related to schizophrenia development [123,127,129–131].

4. Neural stem cell: differentiation and regeneration

Neural stem cells are unspecialized cells capable of differentiating into neuronal or glial populations. Several environmental factors in the neighborhood, especially growth and neurotrophic factors and cytokines, determine cell fate. Binding of growth factors to surface receptors drives intracellular changes that culminate in transcriptional regulation, leading to a decision between maintaining multipotency, undergoing apoptosis or acquiring characteristic transcriptomes of differentiated cells [132].

Ca^{2+} signalling plays a crucial role in integrating cellular responses. $[Ca^{2+}]_i$ level regulation depends both on Ca^{2+} entry through plasma membrane and on its release from internal stores, as discussed previously, and can lead to global or localized micro-domain $[Ca^{2+}]$ increases [133]. In neuronal development, for example, global $[Ca^{2+}]i$ increases have been linked to neurotransmitter phenotype regulation and membrane channel maturation, whilst localized micro-domain $[Ca^{2+}]i$ elevations in growth cones coordinate neurite extension [132].

The process of differentiation requires integration between multiple signalling sources driving specific genetic programs and occur similarly, but not identically, in the developing embryo and in regions of the adult body [29,132]. Neurogenesis in the adult brain is restricted to specific regions and highly coordinated, depending on neural stem cell availability, meaning also self-renewal ability of neural precursor cells, and on efficient, sensitive regulation of neural determination. In the developing embryo, cells from dorsal ectoderm have first to decide between an epidermal or neural fate. These first steps depend on suppression of epidermal inducers, as BMP, and on the onset of $[Ca^{2+}]i$ rises in the cells that will commit to neural lineages in a localized domain. These events involve L-type VGCC Ca^{2+} channel (LTCC)-mediated Ca^{2+} influx, triggering a specific genetic program. In agreement, the absence of this calcium signalling program determines the epidermal fate [134]. LTCC is also important for the differentiation of dental pulp stem cells (DPSCs) into neural cells [135].

In the developing cerebellum, granule cell precursors differentiate upon activation of a homodimeric G protein coupled receptor sensitive to $[Ca^{2+}]$ levels, called calcium-sensing receptor (CaSR). The activation of CaSR *in vivo* also induced homing of the granule cell precursors during differentiation, mainly through the interaction with integrin complexes [136].

In adult neurogenesis, neural stem cells localized in the subventricular zone or the subgranular zone of the hippocampus remain quiescent or proliferating until they receive specific stimuli for migrating and becoming glial cells or neurons [137]. As well as being involved in the neuronal fate choice, $[Ca^{2+}]$ transients also constitute the signalling pathway for astrocytogenesis [138]. Activation of neural precursor cells (NPCs) by astrocytes to trigger a neurogenic response is also mediated by $[Ca^{2+}]_i$ transients. Through upregulation of the Notch signalling pathway after brain injury, calcium waves generated in neighbor astrocytes propagate to NPCs, inducing a neurogenic behavior, including self-renewal and migration of progenitor cells [139].

Much evidence correlates features of $[Ca^{2+}]_i$ oscillations with specific cellular events for differentiation fate determination. Higher frequencies of $[Ca^{2+}]_i$ oscillations, for instance, result in increased differentiation of hippocampus-derived NSCs in neurons [140]. $[Ca^{2+}]_i$ oscillation features are so well established that they are even used to characterize functional neurons and to distinguish neural cell types derived from NSCs [137]. During development, expression patterns of Ca^{2+} channels change, switching from a prevalence of N- and L-types in immature apolar cells to P/Q- and R-type channels in differentiated bi- and multipolar neurons. Furthermore, expression patterns of calcium binding proteins are subject to alterations during differentiation, going from a high parvalbumin level in bipolar neurons to high calretinin and calbindin levels in multipolar neurons [141]. The existence of these patterns points at correlations between the differentiation stage of the cell and the respective Ca^{2+} signalling pattern.

In addition, several channels, effectors and regulators of calcium signalling directly affect neural differentiation. Low-voltage-activated T-type calcium channel (LVAs) blockade in neurosphere cultures of neural progenitor cells (NPCs) decreased migration and neurite extensions in the developing neurons [142], a finding that can be explained by the intracellular Ca^{2+}-dependence of the coupling between the cytoskeleton and integrins.

Expression knockdown of STIM1, a calcium sensor that mediates SOCE, impaired early and late embryonic stem cell differentiation into neural progenitors, neurons or astrocytes, increasing cell death and suppressing the proliferation of neural progenitors. These data show that functional operation of SOCE, leading thus to normal $[Ca^{2+}]_i$ fluxes, is essential for differentiation [143]. Convergent findings showed that pharmacological blockade of SOCE decreased proliferation and self-renewal of NSCs, driving asymmetric division in detriment of symmetric proliferative division and reducing the population of stem cells in the adult brain, and also impaired the ability of subventricular zone cells to form neurospheres in culture [144].

A study conducted in our laboratory revealed that functional P2X7 purinergic receptor (P2X7R) expression, leading to ATP-induced Ca^{2+} fluxes, was higher in mouse embryonic stem cells than in its differentiated counterparts. The blockage of ATP-induced $[Ca^{2+}]_i$ transients by pharmacological inhibition of the P2X7R enhanced progression of retinoic-acid induced neural differentiation of mouse embryonic stem cells, while P2X7R activation resulted in a higher entry of the immature cells into the cell cycle, favoring proliferation of neural progenitors [145].

Stem cell death regulation is also a function of calcium signalling. One of the pathways consists of the modulation of calcium-dependent peptidylarginine deiminase 3 (PAD 3), driving apoptosis-induction factor (AIF) activation, consequent cytoskeleton disassembly and, finally, apoptosis of NSCs [146]. In neurological disorders, the possibility of employing neural stem cells to recover specific regions of the nervous system is highly appealing. For this matter, comprehending how calcium signalling regulates proliferation, migration and differentiation of stem cells is imperative. Further characterization of Ca^{2+} signalling in induce pluripotentstem cells (iPSC) and NPCs suitable for cell culture and expansion will guide the way to transform these findings into therapeutic applications. It is known that *in vitro* differentiation of NPCs shows a time window of Ca^{2+} operative signalling, and these features are predictive of the differentiation stage of the cells [147]. This knowledge may be used to assess suitability of cells for clinical purposes.

Despite the promising use of stem cell therapy coupled to Ca^{2+} signalling modulation, we need to overcome some obstacles like, how, when and where the cells will implant and engraft. For this purpose, we suggest to circumvent the surgical procedure by modulating the endogenous NPCs with the Ca^{2+} signalling modulators. Moreover, another difficulty is the specific assessment of the drugs to NPC, avoiding increasing damage of the niche. The possible alternative, could be coupling the drugs to some nanotechnology tools that would act as carriers of the modulators with specific targeting.

5. Stem cells as a powerful tool to investigate Ca^{2+} signalling in neurological diseases

In neurological disorders, the possibility of employing NSCs to recover specific regions of the nervous system is highly appealing. In addition, modelling of neurological diseases in vitro provides non-invasive and practical tools for basic and translational research. Considering all the points cited above, the main conclusion is that well
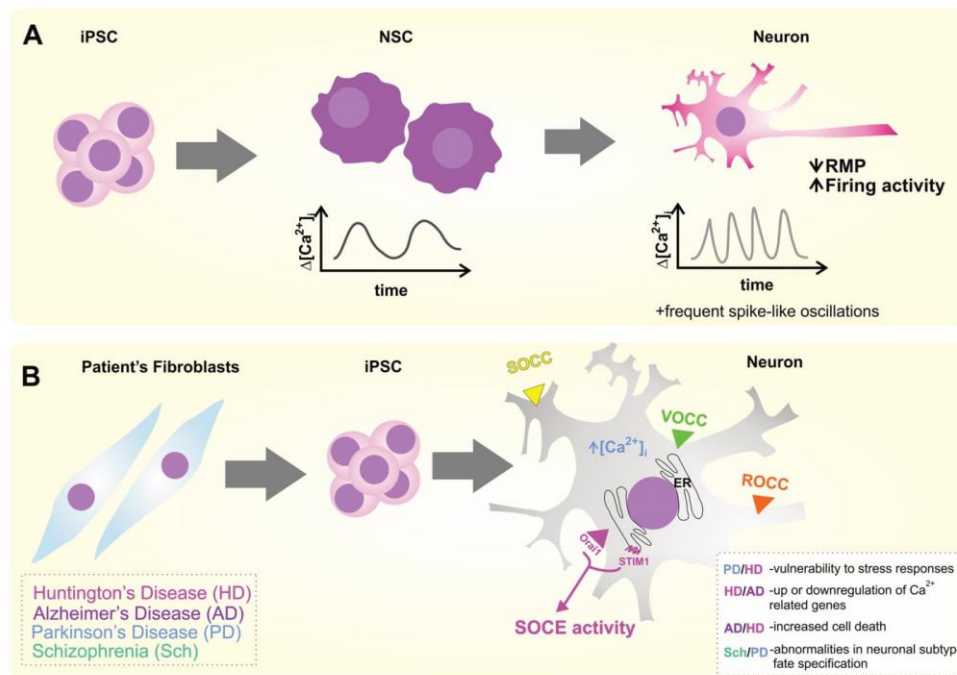
Fig. 2. Ca^{2+} signalling in iPSC derived neurons. **A:** iPSCs derived from adult fibroblasts respond to environmental cues and, upon guided stimuli, become neural precursor cells that will commit to glial or neuronal fate. $[Ca^{2+}]$ oscillations with spike-like morphology are enriched during the progress of neuronal differentiation, and finally reaches a mature pattern compatible with functional neurons. **B:** iPSCs derived from neurological disorders' patients fibroblasts can be fully differentiated, and its derived neurons exhibit distortions, which representative of the disease. In HD patients-derived neurons, SOCE activity is increased and Ca^{2+}-related genes expression is disturbed. In AD patients-derived neurons, besides Ca^{2+}-related gene expression alterations, elevations in glutamate mediated Ca^{2+} entry enhances cell death. In PD patients-derived neurons, $[Ca^{2+}]_i$ is elevated, leading to increased vulnerability to stress responses. In schizophrenia, $[Ca^{2+}]_i$ imbalances drives abnormalities in neuronal subtype fate specification, as well as in PD-derived iPSCs.

characterized $[Ca^{2+}]_i$ pattern footprints would enable distinction of different cell types in distinct differentiation stages.

iPSCs obtained by reprogramming of adult fibroblasts avoid several ethical problems involved in the research use of human embryonic stem cells (hESCs), and provide the opportunity of obtaining patient-derived NPs. This level of specificity and individualization brings research and clinics to another scenario. Thus, functional characterization of iPSCs and their derived NPs and neurons will guide the way to translate these findings not only into more reliable research models, but also into therapeutic applications.

In a study conducted by Prè et al, $[Ca^{2+}]_i$ transients were assessed in forebrain neurons derived from human iPSCs, on days 32 and 55 of differentiation. The neurons became morphologically more complex over time coinciding with more spike-like, more frequent and less durable Ca^{2+} signals, while the resting membrane potential decreased and firing activity of neurons increased. These findings were consistent with immunocytochemistry staining of neuronal markers in differentiated cells and with electrophysiological recordings, pointing at the functionality of iPSC-derived neurons [148].

Looking at specific disease models, neurodegenerative diseases draw special attention. Huntington's disease (HD) patients-derived iPSCs have been proven of being capable to differentiate into medium spiny neurons (MSNs), the neuronal cell type mostly affected by the

progression of the disease. Although it means that it is possible to generate and transplant functional neurons with the exact genetic background of the patient, it also means that the cells will carry the genetic mutations that lead to HD development. As evidence, the transplantation of HD-iPSCs derived NPs into a rat model of HD with unilateral striatal lesion: first, a behavioral recovery was noticed, but weeks later, the pathology signs were again present, highlighting the need for genetic correction [149].

As previously described, HD relates to many disturbances in Ca^{2+} homeostasis. As iPSC-derived neurons are able to present normal Ca^{2+} signalling, this model is as well useful to investigate changes in calcium-related pathways. A study that differentiated HD patients' iPSCs into GABAergic MS-like neurons found increased SOCE activity in three different HD genotypes; moreover, a candidate pharmacological compound was able to restore physiological SOCE currents and displayed neuroprotective action [150]. A transcriptome analysis compared HD-iPSCs-derived and control NSCs, and showed significantly different expression levels for 12 genes involved in Ca^{2+} signalling (the HD IPSC Consortium and Phenotypes 2012). These findings corroborate both the importance of $[Ca^{2+}]_i$ dysregulation in HD pathology and the potential of the model generated based on iPSCs.

The investigation of specific effects of mutations on disease onset and progression, and also on specific pathological alterations, are very

well addressed with the use of patients-derived iPSCs. Reprogrammed fibroblasts derived from patients with GBA-1 mutations, the strongest risk factor for Parkinson's disease (PD) into midbrain dopaminergic neurons, revealed augmented expression of neuronal calcium-binding protein 2 (NECAB2) and elevations in $[Ca^{2+}]_i$ leading to higher vulnerability to stress responses [151]. iPSCs derived neurons also uncovered a correlation between the G2019S mutation in the LRRK2 gene, common in PD, and dysregulations in $[Ca^{2+}]_i$ dynamics [152]. That evidence establishes a link between these mutations and specific features of Ca^{2+} signalling in these neurons.

Using a similar approach, Duan et al. found glutamate exposure-related $[Ca^{2+}]_i$ increases in basal forebrain cholinergic neurons derived from iPSCs of sporadic Alzheimer disease (AD) patients with ApoE3/E4 genotypes, leading to increased cell death [153].

iPSC-derived NPCs are useful not only to investigate neuronal function, but also glial cells. Familial and sporadic AD patients' plus control individual's cells were used to generate iPSC-derived NPs, which were differentiated into astrocytes. The diseased astroglia showed morphological abnormalities, besides ectopic localization of astrocyte markers and an overall atrophic profile [154]. Thus, iPSCs may also be a valuable tool for investigating complex interactions between neurons and glial cells of specific known genotype (Fig. 2).

6. Final remarks

Calcium signalling machinery is crucial for the healthy function of neuronal circuitry, and dysregulation of the pathways is a common feature in many neurological diseases. As we discussed here, it can lead to instability of synapses, mitochondrial dysfunction or induce excitotoxicity through NMDARs. The correction of the dysfunctional components of Ca^{2+} signalling can restore or protect from neurodegeneration. Thus, SOCE, which is the newest pathway described in the field plays important roles in some diseases like AD, HD and PD, although they present slight differences relating to the components. Both HD and PD present depleted ER stores, contrasting to AD (Fig. 2).

Neurogenesis in adult brain is restricted to specific regions and depends on NSCs and NPCs availability. In neurological disorders, the possibility of employing NSCs and NPCs to recover damaged regions is tempting. Thus, the capability to model neurological diseases *in vitro* provides non-invasive and practical tools for the identification of novel therapeutic targets (Fig. 2). The application of iPSC directly in cell therapy is still far to become real, since the drawback of the second clinical trial using iPSC due to the finding of unexpected mutations caused by the reprogramming technique. The potential of cells differentiated from iPSC to generate cancer is still uncertain, opening a new field for research in order to overcome the issue and make cell therapy feasible [155].

As we described above, $[Ca^{2+}]_i$ homeostasis balance is also important for the NSC cell fate phenotypic choice, highlighting the SOCE mechanism that controls the pool size of NSC in the adult brain.

All those findings together points at the $[Ca^{2+}]_i$ machinery as a powerful target to develop novel drugs and strategies to treat neurological diseases.

Acknowledgements

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4.2.5. P2X7 receptor: the central hub of brain diseases. *Frontiers in Molecular Neuroscience*, 2020.

The P2X7 Receptor: Central Hub of Brain Diseases

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The P2X7 receptor is a cation channel activated by high concentrations of adenosine triphosphate (ATP). Upon long-term activation, it complexes with membrane proteins forming a wide pore that leads to cell death and increased release of ATP into the extracellular milieu. The P2X7 receptor is widely expressed in the CNS, such as frontal cortex, hippocampus, amygdala and striatum, regions involved in neurodegenerative diseases and psychiatric disorders. Despite P2X7 receptor functions in glial cells have been extensively studied, the existence and roles of this receptor in neurons are still controversially discussed. Regardless, P2X7 receptors mediate several processes observed in neuropsychiatric disorders and brain tumors, such as activation of neuroinflammatory response, stimulation of glutamate release and neuroplasticity impairment. Moreover, P2X7 receptor gene polymorphisms have been associated to depression, and isoforms of P2X7 receptors are implicated in neuropsychiatric diseases. In view of that, the P2X7 receptor has been proposed to be a potential target for therapeutic intervention in brain diseases. This review discusses the molecular mechanisms underlying P2X7 receptor-mediated signaling in neurodegenerative diseases, psychiatric disorders, and brain tumors. In addition, it highlights the recent advances in the development of P2X7 receptor antagonists that are able of penetrating the central nervous system.

Keywords: P2X7 receptor, neurodegenerative diseases, psychiatric disorders, brain tumor, brain diseases, P2X7 receptor antagonists

INTRODUCTION

The P2X7 Receptor

The investigation of receptors activated by adenosine triphosphate (ATP) has been largely widened since their discovery in 1960s by Geoffrey Burnstock. These receptors are classified into two main types: P1 and P2 receptors. P1 receptors are usually activated by adenosine, have seven transmembrane domains, and are coupled to G proteins. P2 receptors can be divided into two main subtypes, ionotropic P2X receptors and metabotropic P2Y receptors. P2X receptors subunits have just two transmembrane domains and are assembled as homo- or heterotrimers. Such as adenosine-activated P1 receptors, P2Y receptors are coupled to G proteins; however, their ligands are ATP/ADP/ UTP/UDP-glucose (Knight, 2009).

Since the first cloning of the P2X7 receptor from a rat brain cDNA library (Surprenant et al., 1996), it is the most widely investigated purinergic receptor with the largest amount of specific pharmacological tools available (Sluyter and Stokes, 2011).

The *P2RX7* gene is comprised of 13 exons encoding the subunit with 595 amino acids in length that in humans is located at chromosome position 12q24.31 and in mice at chromosome 5. The human *P2RX7* gene is located at the chromosome position also associated with inflammatory and psychiatric disorders (Barden et al., 2006; Lucae et al., 2006). Each one of the three subunits has intracellular amino and carboxyl termini with two hydrophobic transmembrane domains, with a long glycosylated extracellular loop between them, comprising the ATP-binding site. In addition, the P2X7 receptor usually assembles as homotrimer (Sluyter and Stokes, 2011). However, it can also form heteromeric interactions with P2X4 receptor subunits as evidenced in 2007 by Guo et al. (2007) and later confirmed by Schneider et al. (2017).

P2X7 receptor activity is triggered by high concentrations (ranging around 0.05–1 mM) of extracellular adenosine 5′-triphosphate (ATP), mediating the rapid influx of Na^+ and Ca^{2+} and efflux of K^+, and other cations (Burnstock and Kennedy, 2011). Upon long activation, the P2X7 receptor can open pores large enough to allow the passage of organic ions like N-methyl-D-glucamine ($NMDG^+$), $choline^+$ and fluorescent dyes such as $ethidium^+$ and YO-PRO-12^+ (Alves et al., 2014).

Available tools for P2X7 receptor research lack specific agonists. Due to this problem, many literature data need to be carefully analyzed. Studies regarding the activation of P2X7 receptors use agonists, such as ATP and 2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate (Bz-ATP). ATP is a broad agonist for P2X receptors. Bz-ATP is 10–50 times more potent than ATP in activating P2X7 receptors. Besides activating P2X7 receptors, this compound acts as an agonist for P2Y11, P2X1, 2 and 4, and as a weak agonist for P2X5 receptors. Additionally, EC_{50} values for both agonists vary between species. Bz-ATP, for example, activates rat and human P2X7 receptor at 10 times greater concentration than mice P2X7 receptor (Burnstock and Verkhratsky, 2012). As indicated in **Table 1**, some P2X7 receptor antagonists also lack specificity. The widely used Brilliant Blue G (BBG) also antagonizes P2X1, P2X2, P2X3, and P2X4 receptors besides the P2X7 receptor. However, the IC_{50} for the P2X7 receptor is 8–50 times lower compared with other receptors. A-740003, A-438079 and A-804598 are selective for the P2X7 receptor (Burnstock and Verkhratsky, 2012).

Another limitation regarding P2X7 receptors studies is antibody specificity. Available antibodies against the P2X7 receptor are polyclonal, which are prone to cross-reactivity, or monoclonal. Although they did not detect P2X7 receptors in knockout (KO) mice, these monoclonal antibodies failed to consistently and reliably detect and/or block P2X7 receptor signaling pathway in WT mice (Sim et al., 2004; Li et al., 2020). There are at least two P2X7 receptor KO mice commercially available. In the GlaxoSmithKline strain, generated by Chessell et al. (2005), a lacZ transgene and neomycin cassette were inserted into exon 1 (Chessell et al., 2005). These animals express the P2X7K receptor isoform and lack the P2X7A receptor isoform. P2X7K is widely expressed by T lymphocytes, and GlaxoSmithKline P2X7 receptor KO mice possess enhanced P2X7 receptor-mediated responses in T cells. The other available strain from Pfizer, generated by Solle et al. (2001) by inserting a neomycin cassette into exon 13, lacks both P2X7A and K receptor isoforms; however these animals express P2X7 13B and 13C isoforms in the brain and other tissues (Solle et al., 2001; Bartlett et al., 2014). The P2X7 13B isoform was reported to negatively affect P2X7A receptor activity (Masin et al., 2012). Therefore, P2X7 receptor KO mice should be used carefully as a tool to assess P2X7 receptor involvement in brain and inflammation.

Nowadays, P2X7 receptor expression is known to be broadly present throughout diverse tissues and cells, including CNS, such as microglia, oligodendrocytes, Schwann cells, and possibly in astrocytes and neurons. The latter one is still controversial discussed, and various works are still trying to clarify the issue (see Sluyter and Stokes, 2011). Despite several works that demonstrate the presence of P2X7 receptor in neurons (Deuchars et al., 2001; Sperlágh et al., 2002; Wirkner et al., 2005; Yu et al., 2008), its expression and functionality are widely debated (Sim et al., 2004; Anderson and Nedergaard, 2006; Illes et al., 2017; Metzger et al., 2017b). This outlook becomes strengthened when immunoreactivity for this receptor in P2X7 receptor KO strains was detected, evidencing low specificity of anti-P2X7 receptor antibodies (Anderson and Nedergaard, 2006). Recent works with improved methodologies did not find any expression of P2X7 receptors in neurons (Rubini et al., 2014; Kaczmarek-Hajek et al., 2018; Khan et al., 2018). Similarly, the presence of functional P2X7 receptors in astrocytes is also debated c). It is well known, however, that oligodendrocytes and microglia express functional P2X7 receptors (Lord et al., 2015; He et al., 2017; Metzger et al., 2017a; Kaczmarek-Hajek et al., 2018).
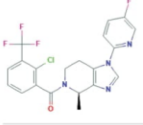
Variants of the P2X7 Receptor

The P2X7 receptor has 10 different alternative splicing isoforms named from P2X7A to P2X7K, the latter has only been identified in rodents (**Figure 1**). The full-length isoform is the P2X7A one. In humans, P2X7B, P2X7H, and P2X7J are the only subunits reported as expressed proteins (Feng et al., 2006; Adinolfi et al., 2010) (**Figure 1**).

The P2X7B isoform is a truncated form, when compared with P2X7A (Cheewatrakoolpong et al., 2005), and assemble as functional channels that cannot form large pores as P2X7A, playing roles in cell proliferation (Adinolfi et al., 2010). The P2X7H is nonfunctional ion channels (Cheewatrakoolpong et al., 2005), whereas the P2X7J can assemble with other splicing variants forming non-functional heterotrimeric receptors (Feng et al., 2006) that are involved in protection against ATP-induced cell death (Feng et al., 2006; Guzman-Aranguez et al., 2017).
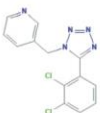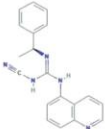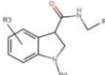
In mice, four splice variants were detected (P2X7B, P2X7C, P2X7D, and P2X7K), besides the canonical P2X7A one. Most of the modifications between isoforms are comprised within the extracellular loop domain. P2X7D and P2X7B can assemble to P2X7A and negatively affect the basal activity of the P2X7 receptor. However, if not assembled to P2X7A, they assemble as receptors forms that show both increased activity and higher sensitivity to agonists (Schwarz et al., 2012; Xu et al., 2012), like the rat P2X7K variant (Nicke et al., 2009). Restricted P2X7 receptor variants present multiple mutations, such as the P2X7 receptor-2 variant that contains H270R and A348T mutations, and the P2X7 receptor-4 variant that has H155Y, H270R, A348T,

TABLE 1 | P2X7 receptor antagonists.

Structure	Compound/IUPAC name	BBB-penetrant	Type	References
	GSK-1482160 (2S)-N-[[2-chloro-3-(trifluoromethyl)phenyl]methyl]-1-methyl-5-oxopyrrolidine-2-carboxamide	Yes	Preferential P2X7 receptor antagonist	Territo et al., 2017; Kim et al., 2019
	GSK-314181A N-(1-adamantylmethyl)-5-[[(3R)-3-aminopyrrolidin-1-yl]methyl]-2-chlorobenzamide;hydrochloride	Yes	Preferential P2X7 receptor antagonist	Broom et al., 2008; Kim et al., 2019
	Compound 16 (GSK) (2,4-dichlorophenyl)-methylazanide	Yes	Preferential P2X7 receptor antagonist	Beswick et al., 2010; Kim et al., 2019
	JNJ-54175446 [2-chloro-3-(trifluoromethyl)phenyl]-[(4R)-1-(5-fluoropyrimidin-2-yl)-4-methyl-6,7-dihydro-4H-triazolo[4,5-c]pyridin-5-yl]methanone	Yes	Preferential P2X7 receptor antagonist	Letavic et al., 2017; Kim et al., 2019
	JNJ-55308942 (S)-(3-fluoro-2-(trifluoromethyl)pyridin-4-yl)(1-(5-fluoropyrimidin-2-yl)-6-methyl-1,4,6,7-tetrahydro-5H-[1,2,3]triazolo[4,5-c]pyridin-5-yl)methanone	Yes	Non-selective P2X7 receptor antagonist (also binds to P2X1, P2X2, P2X3, P2X2/3, and P2X4 receptors)	Chrovian et al., 2018
	JNJ-42253432 2-methyl-N-([1-(4-phenylpiperazin-1-yl)cyclohexyl]methyl)-1,2,3,4-tetrahydroisoquinoline-5-carboxamide	Yes	Preferential P2X7 receptor antagonist	Letavic et al., 2013; Lord et al., 2014
	JNJ-47965567 N-[[4-(4-phenylpiperazin-1-yl)oxan-4-yl]methyl]-2-phenylsulfanylpyridine-3-carboxamide	Yes	Preferential P2X7 receptor antagonist	Bhattacharya et al., 2013; Letavic et al., 2013; Kim et al., 2019
	JNJ-54166060 [2-chloro-3-(trifluoromethyl)phenyl]-[(4R)-1-(5-fluoropyridin-2-yl)-4-methyl-6,7-dihydro-4H-imidazo[4,5-c]pyridin-5-yl]methanone	Yes	Preferential P2X7 receptor antagonist	Swanson et al., 2016; Kim et al., 2019
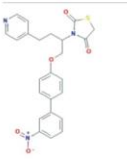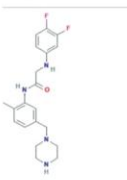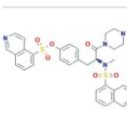
(Continued)

TABLE 1 | Continued

Structure	Compound/IUPAC name	BBB-penetrant	Type	References
	A-438079 3-[[5-(2,3-dichlorophenyl)tetrazol-1-yl]methyl]pyridine	Yes	Preferential P2X7 receptor antagonist	Nelson et al., 2006; Kim et al., 2019
	A-740003 N-[1-[(Z)-[(cyanoamino)-(quinolin-5-ylamino)methylidene]amino]-2,2-dimethylpropyl]-2-(3,4-dimethoxyphenyl)acetamide	Yes	Preferential P2X7 receptor antagonist	Honore et al., 2006; Kim et al., 2019
	A-804598 1-cyano-2-[(1S)-1-phenylethyl]-3-quinolin-5-ylguanidine	Yes	Preferential P2X7 receptor antagonist	Donnelly-Roberts et al., 2009; Able et al., 2011; Kim et al., 2019
	A-839977 1-(2,3-dichlorophenyl)-N-[(2-pyridin-2-yloxyphenyl)methyl]tetrazol-5-amine	Yes	Preferential P2X7 receptor antagonist	Honore et al., 2009; Kim et al., 2019
	AFC-5128 indol-3-carboxamide derivative, chemical nomenclature disclosed	Yes	Preferential P2X7 receptor antagonist	Fischer et al., 2016
	Brilliant blue G (BBG) 3-[[4-[(E)-[4-(4-ethoxyanilino)phenyl]-[4-[ethyl-[(3-sulfonatophenyl)methyl]azaniumylidene]-2-methylcyclohexa-2,5-dien-1-ylidene]methyl]-N-ethyl-3-methylanilino]methyl]benzenesulfonate	Yes	Non-selective P2X7 receptor antagonist (also binds to P2X1, P2X2, P2X3 and P2X4 receptors)	Savio et al., 2018; Kim et al., 2019
	CE-224, 535 2-chloro-N-[(1-hydroxycycloheptyl)methyl]-5-[4-[(2R)-2-hydroxy-3-methoxypropyl]-3,5-dioxo-1,2,4-triazin-2-yl]benzamide	No	High selective P2X7 receptor antagonist (500-fold over P2X1 and P2Y1 receptors)	Savall et al., 2015; Kim et al., 2019
	AZD9056 N-(1-adamantylmethyl)-2-chloro-5-[3-(3-hydroxypropylamino)propyl]benzamide	No	Preferential P2X7 receptor antagonist	Bhattacharya, 2018; Kim et al., 2019

(Continued)

TABLE 1 | Continued

Structure	Compound/IUPAC name	BBB-penetrant	Type	References
	AZ-10606120 2-(1-adamantyl)-N-[2-[2-(2-hydroxyethylamino)ethylamino]quinolin-5-yl]acetamide;dihydrochloride	Not found	Negative allosteric modulator of the human P2X7 receptor.	Kim et al., 2019
	AZ-11645373 3-[1-[4-(3-nitrophenyl)phenoxy]-4-pyridin-4-ylbutan-2-yl]-1,3-thiazolidine-2,4-dione	Not found	Preferential P2X7 receptor antagonist (500 times less effective in rat than in human P2X7 receptors)	Kim et al., 2019
	GW791343 2-(3,4-difluoroanilino)-N-[2-methyl-5-(piperazin-1-ylmethyl)phenyl]acetamide	Not found	Negative activity modulator of human P2X7 receptors, positive activity modulator of rat P2X7 receptors	Kim et al., 2019
	KN-62 [4-[(2S)-2-[isoquinolin-5-ylsulfonyl(methyl)amino]-3-oxo-3-(4-phenylpiperazin-1-yl)propyl]phenyl] isoquinoline-5-sulfonate	Not found	Preferential human P2X7 receptor antagonist, however with low affinity to rat P2X7 receptors	Kim et al., 2019

BBB: blood brain barrier; IUPAC: International Union of Pure and Applied Chemistry.

and Q460R mutations (Stokes et al., 2010). These variants in heterologous expression cells also exhibited larger agonist-induced ion currents and dye uptake with a similar agonist sensitivity (Jiang et al., 2013).

Some alternative splicing isoforms of P2X7 receptor show diverse downstream signaling properties. Moreover, P2X7 receptor function varies among human individuals because there are some polymorphisms that can result in loss- or gain-of-function (**Figure 2**). Single nucleotide polymorphisms (SNPs) are widespread in the human P2X7 receptor; some of them are non-synonymous, meaning that there is a change in the amino acid sequence, generating a point mutation. Some of those mutations are related to altered susceptibility to various diseases, shedding new light on the underlying disease mechanisms (Jiang et al., 2013). In this article, we review SNPs involved in Alzheimer's disease (AD) (rs208294, rs3751143), Parkinson's disease (PD) (rs3751143), multiple sclerosis (MS) (rs208294, rs28360457), depressive disorder (rs7958311, rs2230912), anxiety (rs208294, rs2230912), and bipolar disorder (BD) (rs208294, rs1718119, rs2230912, rs3751143) (**Figure 2**).

P2X7 Receptor Function

P2X7 receptor activation induces a number of well-established downstream signaling events in various human cell types. The opening of the channel leads to an increase in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_i$), responsible for a number of P2X7 receptor-induced responses, as AKT activation in astrocytes (Jacques-Silva et al., 2004). Phospholipase (PL) C and A2 (Andrei et al., 2004), src kinase, p38, acid sphingomyelinase (Bianco et al., 2009), caspase 1 (Keller et al., 2008), and gasdermin (Evavold et al., 2018) are involved in P2X7 receptor intracellular signaling.

P2X7 receptor activity mediates cell proliferation and death, rapid and reversible phosphatidylserine exposure, membrane blebbing, release of microparticles and exosomes and multinucleated cell formation, as well as the formation of reactive oxygen and nitrogen species (Sluyter and Stokes, 2011).

P2X7 Receptor in Neuroinflammation

The P2X7 receptor is highly expressed in microglial cells (Lord et al., 2015; He et al., 2017). In healthy tissues, the concentration of extracellular ATP is low at the nanomolar range (Falzoni et al., 2013). Conversely, under stress and cellular damage, the ATP concentration increases considerably, resulting in P2X7 receptor activation. Therefore, it is hypothesized that P2X7 receptor acts as a silent receptor once its activation only occurs in pathological states when there is a rise of extracellular ATP concentrations (Bhattacharya and Biber, 2016).

FIGURE 1 | P2X7 receptor splicing variants. The P2X7 receptor has 10 different isoforms derived from alternative splicing and mutations of the 13 exons of the gene. The P2X7A isoform is the native form, expressed in every mammal species. The detected alterative isoforms in humans are P2X7B, H and J, while in rodents, these are P2X7B, C, D and K. The mutations that lead to a stop codon insertion, originate a shortened P2X7 receptor at the carboxy-terminal domain and cannot form pores that induce cell death. P2X7G and H present a copy of exon 3 (N3) near the amino-terminal. Known basic functions for each isoform are described at the right site of the panel. Aminoterminal (NH⁺), Carboxyterminal (COO⁻), Transmembrane passage 1 (TM1), Transmembrane passage 2 (TM2).

In high concentrations, extracellular ATP can act as a damage-associated molecular patterns (DAMPs) and activate P2X7 receptor (Falzoni et al., 2013). DAMP signal activates the transcription factor NF-κB in the nucleus, which consequently promote the upregulation of proinflammatory cytokines, pro-IL-1β and pro-IL-18, and NLRP3 protein (Jo et al., 2016). Although the precise mechanism is not completely understood, P2X7 receptor it is one of the most potent activators of the NRLP3-associated inflammasome (He et al., 2017). P2X7 receptor activation induces K^+ efflux, which is needed for efficient NLRP3 inflammasome activation (Gustin et al., 2015). NLRP3 inflammasome trigger the activation of caspase-1, which causes the maturation of IL-1β and IL-18 and, consequently, increasing proinflammatory cytokine release (Bernier, 2012; Jo et al., 2016; He et al., 2017; Bhattacharya et al., 2018). This signaling appears to be in functional in microglia and not astrocytes (Gustin et al., 2015). Additionally, the P2X7 receptor may also stimulate the release of TNF, IL-6, CCL2, CCL3, and CXCL2 (Suzuki et al., 2004; Kataoka et al., 2009; Shiratori et al., 2010; Shieh et al., 2014).
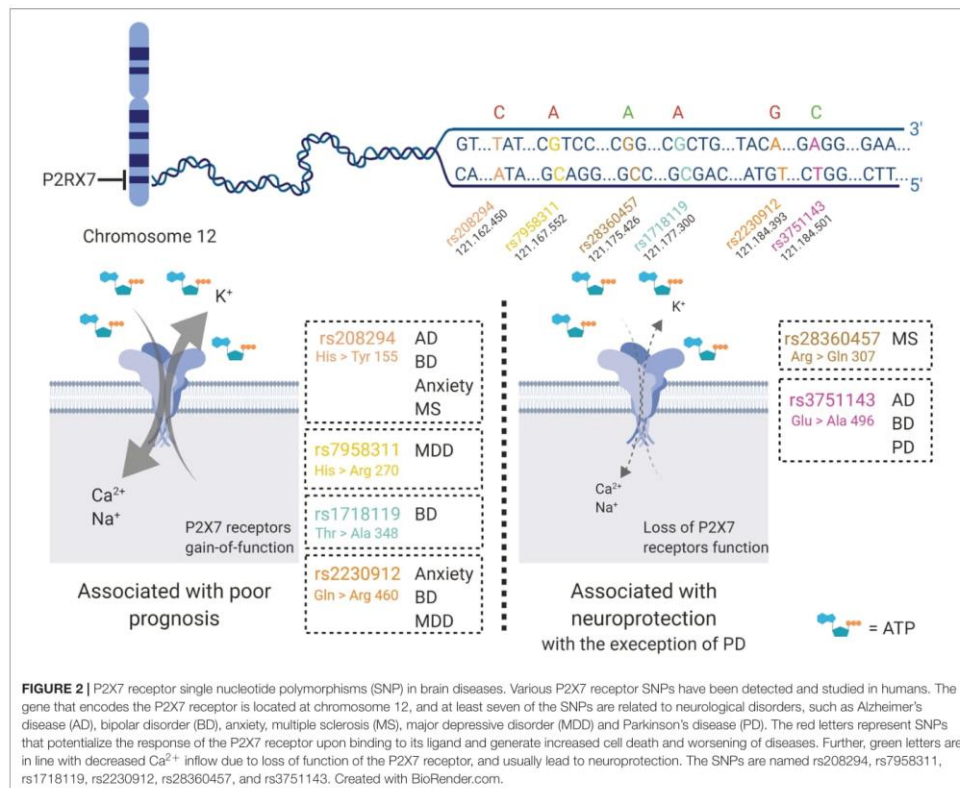
It is clear that the P2X7 receptor can modulate the neuroinflammation induced by LPS, once P2X7 receptor blockade may reduce inflammatory mediators release (Bianco et al., 2006; Choi et al., 2007; Monif et al., 2009; He et al., 2017; Yang et al., 2018). Some works showed that LPS enhanced P2X7 receptor expression (Choi et al., 2007; Yang et al., 2018), whereas other studies reported downregulation of P2X7 receptor expression (Bianco et al., 2006; He et al., 2017). Similarly to the LPS-induced effects, P2X7 receptor overexpression was sufficient to trigger microglial activation in primary microglia derived from hippocampus (Monif et al., 2009). Interestingly, a recent study evidenced that the selective P2X7 receptor antagonist, JNJ-55308942, inhibited neuroinflammation development induced in different rodent models by LPS, BCG or chronic stress (Bhattacharya et al., 2018). Recently, efforts were made to detect *in vivo* neuroinflammation. Therefore, radioligands targeting P2X7 receptor were used as a tool to identify brain areas undergoing inflammatory processes. [18F]-JNJ-64413739 and 11C-GSK1482160 were promising in detecting areas of neuroinflammation upon LPS-stimulation of in rodents (Territo et al., 2017; Berdyyeva et al., 2019).

One of the possible pathways for ATP release is from dying cells. Interestingly, diseases that present degeneration of neural cells, as neurodegenerative diseases, psychiatric disorders, and brain tumors, as presented below, may present high local concentrations of extracellular ATP and stimulate pathophysiological P2X7 receptor activity. In view of that, here, we provide evidence that AD, PD, MS, depression, and brain tumors present increased P2X7 receptor expression. P2X7 receptor signal amplification in these diseases is proposed.

P2X7 RECEPTOR ROLES IN NEURODEGENERATIVE DISEASES

Purinergic receptors play a significant role in neurodegenerative diseases (Oliveira-Giacomelli et al., 2018). P2X7 receptors participate in neurodegenerative, neuroinflammatory and neurogenic processes, tightly related to disease development and repair.

FIGURE 2 | P2X7 receptor single nucleotide polymorphisms (SNP) in brain diseases. Various P2X7 receptor SNPs have been detected and studied in humans. The gene that encodes the P2X7 receptor is located at chromosome 12, and at least seven of the SNPs are related to neurological disorders, such as Alzheimer's disease (AD), bipolar disorder (BD), anxiety, multiple sclerosis (MS), major depressive disorder (MDD) and Parkinson's disease (PD). The red letters represent SNPs that potentialize the response of the P2X7 receptor upon binding to its ligand and generate increased cell death and worsening of diseases. Further, green letters are in line with decreased Ca^{2+} inflow due to loss of function of the P2X7 receptor, and usually lead to neuroprotection. The SNPs are named rs208294, rs7958311, rs1718119, rs2230912, rs28360457, and rs3751143. Created with BioRender.com.

Alzheimer's Disease

Alzheimer's disease is the most common form of dementia in the elderly population (Ballard et al., 2011; Beinart et al., 2012), representing a serious public health problem. Recent estimative indicates that approximately 50 million people have AD worldwide, and this number is expected to reach 132 million by 2050 (Alzheimer's Association, 2015). Processes that trigger AD may start decades before the onset of initial symptoms of dementia (Goedert and Spillantini, 2006; De Felice, 2013), reinforcing the importance of sensitive diagnostic tools for more effective therapeutic interventions.

The main clinical symptom in AD is the cognitive decline, which begins with recent memory lapses, and proceeds with progressively intensified memory loss to total physical dependence. Familial AD (∼5% of all cases) is more severe and initiates earlier than the sporadic form, affecting people from 40 years of age on. Most patients are sporadic cases, presenting AD symptoms from 65 years of life on, and aging is indicated as the leading risk factor for the disease (Evans et al., 1989). The pathophysiologic generation of the neurotoxic β-amyloid oligomers (AβO) by sequential amyloid precursor protein (APP) proteolysis is involved in the development of AD. Familial AD has been directly related to mutations in the genes of APP and presenilin 1 and 2 (Levy-Lahad et al., 1995; Sherrington et al., 1995). The etiology of AD is an association between genetic and environmental factors (Selkoe, 2004; Roberson and Mucke, 2006) which turns disease treatment more difficult. Indeed, the drugs currently available to treat AD have only palliative effects and consist of acetylcholinesterase inhibition to optimize cholinergic activity (Knapp et al., 1994; Rogers and Friedhoff, 1996; Trinh et al., 2003), and the NMDA receptor antagonist memantine (Cosman et al., 2007; Lipton, 2007; Parsons et al., 2007; Xia et al., 2010). Therefore, the development of more effective drugs for AD treatment is needed.

There is evidence that inflammation plays a vital role in AD (Lucin and Wyss-Coray, 2009), as well as in the modulation of neurogenesis (Mishra et al., 2015). Interestingly, there is a significant influence of microglia in both processes (Nunan et al., 2014; De Lucia et al., 2016). AβO also activates

microglia, resulting in secretion of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and IL-1β (Ledo et al., 2013, 2016). Microglial activation may not only compromise their clearance ability (Heneka et al., 2010) but also, surprisingly, contribute to the propagation of AβO in the cerebral parenchyma (Joshi et al., 2014). Interestingly, the P2X7 receptor is involved in these features and in AD as discussed in the following.

Increased P2X7 receptor expression and activation have been involved in the progression of several neurodegenerative diseases, including AD (Savio et al., 2018). Accordingly, P2X7 receptor expression is increased in the brain of AD patients and appears to be concentrated in areas of higher density of amyloid plaques, co-localized with activated microglia (McLarnon et al., 2006). P2X7 receptors expression are also upregulated in the hippocampus of two animal models of AD, such as transgenic mice that express the human APP bearing the Swedish mutation (K670N/M671L) (Parvathenani et al., 2003) and rats injected with amyloid-β peptide (Aβ) 1-42 (1 nmol) into the hippocampus (McLarnon et al., 2006) (**Figure 3**).

Further, the involvement of two P2X7 receptor SNPs were investigated in AD patients and compared to age-matched non-demented elderly, the 1513A > C (rs3751143) and 489C > T (rs208294) (Sanz et al., 2014). This study showed that the presence of the 1513C allele and the absence of the 489C allele (i.e., the presence of both SNPs) decreased the probability of having AD by about four-fold versus the reference subgroup (Sanz et al., 2014). The 1513A > C substitution is associated to the loss of P2X7 receptor function and should confer an "anti-inflammatory" phenotype (Gu et al., 2001). On the other hand, the 489C > T SNP causes a gain of function of this receptor, which may potentiate P2X7 receptor-induced phagocytosis, and subsequent Aβ elimination (Cabrini et al., 2005; Sluyter and Stokes, 2011) (**Figure 2**). Therefore, such mutations may be neuroprotective against AD development (Sanz et al., 2014).
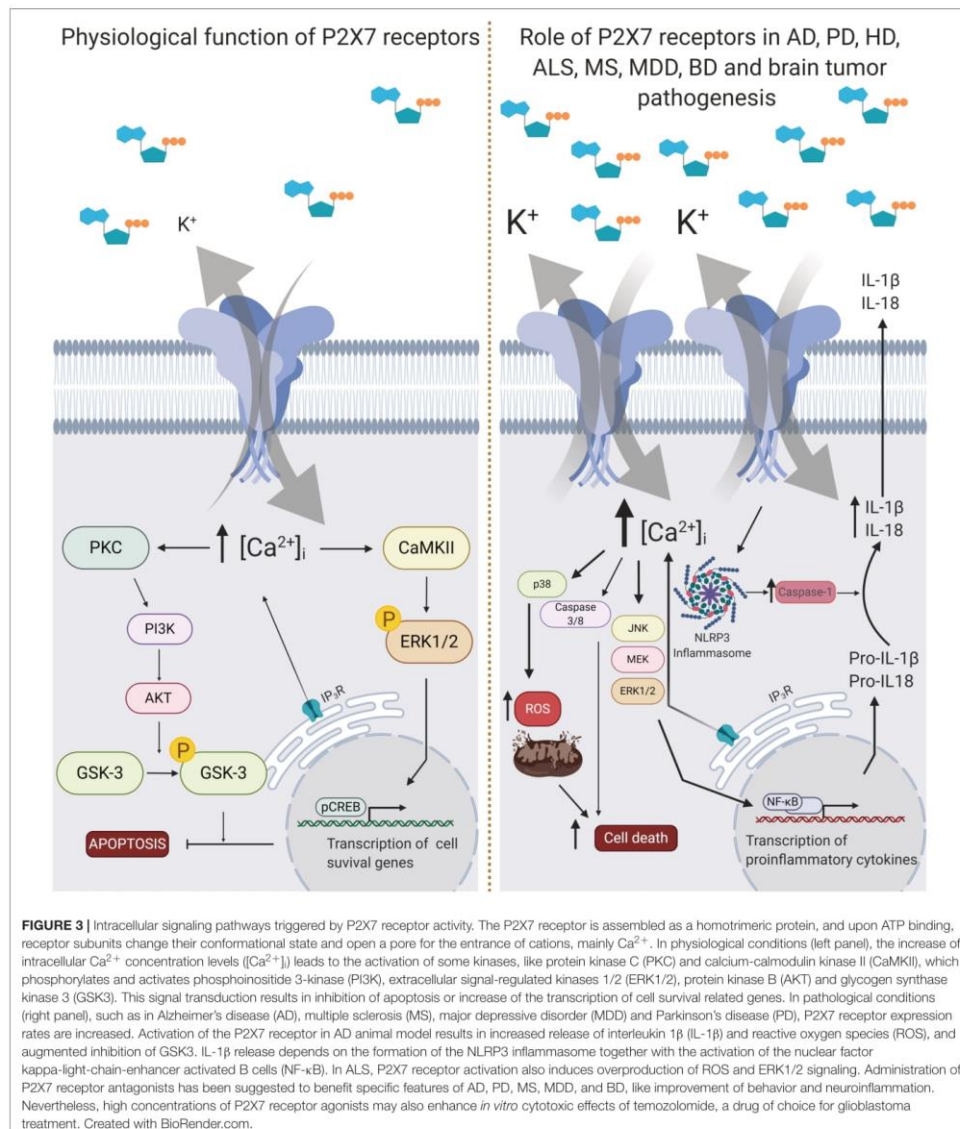
Several studies support the idea that the prolonged activation of P2X7 receptor may result in increased secretion of pro-inflammatory cytokines (such as IL1-β and IL-18) and reduced phagocytic capacity, leading to neuronal damage (Skaper et al., 2006; Sanz et al., 2009; Lee et al., 2011). In accordance with this proposal, injection of fibrillar amyloid-β peptide (fAβ1-42) into the dentate gyrus of the hippocampus enhanced microglial reactivity, astrogliosis and leakiness of the blood-brain barrier (Ryu and McLarnon, 2008a,b). Interestingly, a pronounced increase of P2X7 receptor immunoreactivity was detected in astrocytes and microglia, but not in neurons (McLarnon et al., 2006; Ryu and McLarnon, 2008a). Aβ1-42 treatment also induced ATP release, $[Ca^{2+}]_i$ enhancement and IL-1β secretion in primary microglial cell cultures prepared from wild-type, but not from P2X7 receptor KO mice (Sanz et al., 2009). Intra-hippocampal injection of Aβ1-42 caused a large accumulation of IL-1β in wildtype, but not in P2X7 receptor KO mice (Sanz et al., 2009). Treatment with Bz-ATP increased IL-1β secretion from human microglia cells pre-activated with Aβ1–42, which was completely reversed following pre-incubation with oxidized ATP, P2X7 receptor antagonist (Rampe et al., 2004). This response may be mediated by P2X7 receptors, since the treatment with the selective P2X7 receptor antagonist A-740003 blocked the release of IL-1β induced by ATP treatment of microglial cells from rat cortex incubated with serum amyloid A (**Figure 3**).

Further evidence indicated that P2X7 receptor activation may also induce neuronal damage in AD through the production of reactive oxygen species (ROS). In microglial cultures, Aβ1-42 induced ATP release leading to the production of ROS via P2X7 receptor activation (Soo et al., 2007). A catalytic subunit of NADPH oxidase, which catalyzes the production of ROS, was detected in P2X7 receptor-positive microglial cells in the cerebral cortex of 6-months-old APP/PS1 mice, a double transgenic mice commonly used to study familial AD (Lee et al., 2011). Moreover, postsynaptic density 95-positive dendrites showed significant damage in P2X7 receptor-positive regions in the cerebral cortex of these animals (Lee et al., 2011). Up-regulation of P2X7 receptor expression and ROS production in microglia cells were temporally correlated with Aβ increase and synaptotoxicity in this animal model, since it occurs around the age of 6 months (Lee et al., 2011).

Studies demonstrated that P2X7 receptor activation interferes with processing of APP. APP is proteolytically processed by β- and γ-secretases to release Aβ, the main component of senile plaques found in the brains of AD patients (Zhang et al., 2011). Alternatively, APP can be cleaved by α-secretase, leading to the formation of the nonpathogenic amyloid-α peptide (Aα) (Zhang et al., 2011). In two different cellular lines (HEK293T and neuroblastoma N2a), inhibition of either constitutive expression or overexpression of the P2X7 receptor increased α-secretase activity through inhibition of glycogen synthase kinase 3 (GSK-3) (León-Otegui et al., 2011; Diaz-Hernandez et al., 2012; Miras-Portugal et al., 2015). In addition, systemic administration of P2X7 receptor antagonists in APP$_{SweInd}$ mice, a transgenic animal that expresses the human APP bearing both the Swedish (K670N/M671L) and the Indiana (V717F) mutations, decreased the number of hippocampal amyloid plaques (Diaz-Hernandez et al., 2012; Miras-Portugal et al., 2015). This reduction is correlated with a decrease in GSK-3 activity and consequent increase of α-secretase activity, leading to non-amyloidogenic APP processing (Diaz-Hernandez et al., 2012; Miras-Portugal et al., 2015).

However, results from Delarasse et al. (2011) showed the opposite effect: P2X7 receptor stimulation may enhance α-secretase activity. In this work, four different cell lines (mouse and human neuroblastoma cells, primary murine astrocytes and neural progenitor cells) incubated with ATP or Bz-ATP had activated enzymatic cascades that triggered α-secretase activity, leading to increased levels of Aα, while Aβ was undetectable (Delarasse et al., 2011). Moreover, this study provides evidence to support the idea that ATP- or Bz-ATP-mediated Aα release is mediated by P2X7 receptor activation: (1) three pharmacological inhibitors of P2X7 receptor blocked the release of Aα mediated by Bz-ATP; (2) inhibition of P2X7 receptor synthesis by RNA interference reduced Aα production; and (3) stimulation by Bz-ATP of mouse primary astrocytes and neural progenitor cells from P2X7 receptor-deficient mice did not induce Aα release, while it did in cells derived from wild type animals (Delarasse et al., 2011). Despite such interesting data, it

FIGURE 3 | Intracellular signaling pathways triggered by P2X7 receptor activity. The P2X7 receptor is assembled as a homotrimeric protein, and upon ATP binding, receptor subunits change their conformational state and open a pore for the entrance of cations, mainly Ca^{2+}. In physiological conditions (left panel), the increase of intracellular Ca^{2+} concentration levels ($[Ca^{2+}]_i$) leads to the activation of some kinases, like protein kinase C (PKC) and calcium-calmodulin kinase II (CaMKII), which phosphorylates and activates phosphoinositide 3-kinase (PI3K), extracellular signal-regulated kinases 1/2 (ERK1/2), protein kinase B (AKT) and glycogen synthase kinase 3 (GSK3). This signal transduction results in inhibition of apoptosis or increase of the transcription of cell survival related genes. In pathological conditions (right panel), such as in Alzheimer's disease (AD), multiple sclerosis (MS), major depressive disorder (MDD) and Parkinson's disease (PD), P2X7 receptor expression rates are increased. Activation of the P2X7 receptor in AD animal model results in increased release of interleukin 1β (IL-1β) and reactive oxygen species (ROS), and augmented inhibition of GSK3. IL-1β release depends on the formation of the NLRP3 inflammasome together with the activation of the nuclear factor kappa-light-chain-enhancer activated B cells (NF-κB). In ALS, P2X7 receptor activation also induces overproduction of ROS and ERK1/2 signaling. Administration of P2X7 receptor antagonists has been suggested to benefit specific features of AD, PD, MS, MDD, and BD, like improvement of behavior and neuroinflammation. Nevertheless, high concentrations of P2X7 receptor agonists may also enhance *in vitro* cytotoxic effects of temozolomide, a drug of choice for glioblastoma treatment. Created with BioRender.com.

is relevant to emphasize that APP processing depends on the abundance of this protein at the specific cellular model and, in this case, the equilibrium between the different proteolytic pathways could be unbalanced, which could explain the contrast with the results obtained by other authors (León-Otegui et al., 2011; Diaz-Hernandez et al., 2012; Miras-Portugal et al., 2015).

Therefore, the roles of P2X7 receptors in α-secretase activity and APP processing are controversial and should be further investigated. In addition to the aforementioned effects mediated by P2X7 receptors, these receptors have also been involved in memory and cognition impairment, key symptoms of AD frequently attributed to Aβ deposits and neurofibrillary tangles, which spread from the trans-entorhinal and hippocampal regions to the primary areas of the neocortex (Raskin et al., 2015). In accordance with the detrimental role of P2X7 receptor activation in AD, systemic administration of a P2X7 receptor antagonist, BBG, diminished spatial memory impairment and cognitive deficits along with reduced loss of filopodia and spine density induced by the injection of soluble Aβ1-42 into the hippocampal CA1 region of mice, an animal model of AD (Chen et al., 2014). BBG also inhibits, at a lesser extent, P2X4 receptors, which could be responsible for the observed neuroprotective effects. Knockdown of the P2X4 receptor attenuated Aβ1-42-induced neuronal death in neurons primary culture, whereas induction of P2X4 receptor expression in a neuronal cell line that does not express P2 receptors enhanced the toxic effect of Aβ1-42 (Varma et al., 2009).

However, other authors observed that P2X7 receptor inhibition may induce memory deficits. For instance, P2X7 receptor KO mice displayed spatial memory impairment in the Y-maze test, despite their performances in the object recognition task remained unaltered (Labrousse et al., 2009). Additionally, P2X7 receptor KO mice or wild type animals treated with A-438079 presented increased contextual fear recall and impaired acquisition of extinction in mice (Domingos et al., 2018). The treatment with A-740003 elicited dose-dependent impairments in memory acquisition, consolidation and retrieval in rats, whereas P2X7 receptor deletion hampered the aversive memory processes of mice exposed to the contextual fear-conditioning task (Campos et al., 2014). The obtained results indicate that P2X7 receptor inhibition induces memory impairment associated to anxiogenic-like responses. At this point, it is important to highlight that such studies were not conducted in an animal model of AD, but in tests used to evaluate memory and anxiety-related behaviors. The opposite effect observed in an animal model of AD is understandable since experimental conditions were different.

Altogether, literature data indicates that P2X7 receptor inhibition: (1) ameliorates neuronal damage induced by both neuroimmune response activation and ROS production; (2) modulates α-secretase activity and non-amyloidogenic APP processing, in a non-elucidated manner; and (3) attenuated spatial memory impairment and cognitive deficits in an animal model of AD. These results support that P2X7 receptor antagonism may be a possible strategy for AD treatment.

Parkinson's Disease

Parkinson's disease is a neurodegenerative disease that affects more than 1% of the world's elderly population (between 60 and 80 years old) (de Lau and Breteler, 2006). Despite its high incidence, PD etiology is still poorly understood. Dopaminergic neurons of the nigrostriatal pathway undergo neurodegeneration, accompanied by neuroinflammation and oxidative stress. The appearance of protein aggregates formed by α-synuclein aggravating the disease state is also one of the hallmarks of the disease, although it is not the main cause of dopaminergic neuron death (Hornykiewicz, 1966; Hughes et al., 1992; de Lau and Breteler, 2006).

Patients with PD have characteristic symptoms, such as shaking palsy, resting tremor and bradykinesia, as well as non-motor symptoms, including cognitive impairment and mood and sleep disorders (Thenganatt and Jankovic, 2014). Current treatments consisting of remission of symptoms trigger several adverse effects that compromise the quality of life of the individual. There is no known cure for the disease, highlighting the importance of elucidating the mechanisms involved in the disease and possible therapeutic targets (Hornykiewicz, 2002).

In humans, genetic predisposition to PD development was identified in patients carrying P2X7 receptor polymorphisms. In a Han Chinese population, the P2X7 receptor polymorphism rs3751143 (Glu496Ala) was identified as a risk factor for PD (Liu et al., 2013) (**Figure 2**).

Animal models of PD show that the P2X7 receptor is involved in disease development, especially in microglial cell activation. In an animal model of nigrostriatal injury induction by 6-OH dopamine (6-OHDA), a toxic dopamine analog, striatal gene expression of the P2X7 receptor gradually increased over 5 weeks after injury (Oliveira-Giacomelli et al., 2019). Neuroprotective effects of P2X7 receptor antagonism were observed after pretreating animals with A-438079. This treatment prevented the decrease in striatal dopamine stocks triggered by 6-OHDA injection. However, this effect was not accompanied by a reduction of dopaminergic neuron death, indicating that P2X7 receptor inhibition acts on axonal dopamine stores (Marcellino et al., 2010) (**Figure 3**).

Similar results were obtained with BBG treatments. When administered prior to induction of the 6-OHDA injury, intracerebroventricular injection of BBG also protected against decreasing striatal dopamine levels and reduced oxidative stress, mitochondrial dysfunction and apoptosis (Kumar et al., 2017). Treatment with BBG (45 mg/kg) in rats prevented the reduction of striatal and nigral dopamine levels, decreased astrogliosis, striatal microgliosis, and the number of apomorphine-induced rotations (Carmo et al., 2014). Controversially, Hracskó et al., 2011 showed that P2X7 receptor KO animals are equally susceptible to dopaminergic neuron death induction by MPTP (Hracskó et al., 2011). In this study, the Pfizer KO mouse strain was used, known to express P2X7 13C and 13B receptors in the brain (Bartlett et al., 2014).

Additionally, it is suggested that P2X7 receptor inhibition may also promote neuroregeneration of dopaminergic neurons when given 1 week after 6-OHDA-induced injury (Ferrazoli et al., 2017; Oliveira-Giacomelli et al., 2019). Administration of BBG (50 mg/kg) in rats during 7 days, starting 1 week after injury, augmented the number of substantia nigra dopaminergic neurons (Ferrazoli et al., 2017). Likewise, BBG (75 mg/kg) treatment also regenerated striatal dopaminergic fibers. This effect was accompanied by decreased microglial activation in the substantia nigra (Oliveira-Giacomelli et al., 2019) (**Figure 3**).

Treatment of neuronal-differentiated SH-SY5Y cells, an *in vitro* model of dopaminergic neurons, with BBG protected cells from 6-OHDA-induced synaptotoxicity and death (Carmo et al., 2014; Oliveira-Giacomelli et al., 2019). In addition, assays with wild-type and α-synuclein mutants of microglial cells showed that α-synuclein activated microglial P2X7 receptors, inducing NADPH oxidase, modulating the PI3K/AKT signaling pathway and increasing oxidative stress (Jiang et al., 2015). Subsequently, it has been reported that this α-synuclein-promoted effect on microglial cells *in vitro* also involves the stimulation of glutamatergic excitotoxicity (Dos-Santos-Pereira et al., 2018).

Overall, P2X7 receptor inhibition presents neuroprotective and neuroregenerative effects in cellular and animal models of PD. This effect involved anti-inflammatory actions and modulation of the microglial activation state and cytokine release. However, most of these studies used BBG as a tool to assess P2X7 receptor antagonism. Therefore, we cannot discard that P2X4 receptors could be partially responsible for neuroprotective and/or neuroregenerative effects in PD's models (Ase et al., 2015). P2X4 receptor inhibition did not prevent 6-OHDA-induced cell death in SH-SY5Y cell culture (Oliveira-Giacomelli et al., 2019). This result indicates that P2X4 receptor antagonism is not the main mechanism of neuroprotective effect of BBG treatment. On the other hand, there is no reported study of P2X4 receptor antagonism inducing neuroregenerative effects. Thus, P2X4 receptor antagonism could be partially responsible for the regeneration of dopaminergic neurons in the animal model of PD induced by 6-OHDA. In conclusion, P2X7 receptor is an interesting research topic and possible target for PD.

Huntington's Disease

Huntington's disease (HD) is a dominant hereditary disease caused by a mutation in IT15 gene that encodes huntingtin protein (Htt). Abnormal elongation of the (CAG)n repeats localized in 5′ coding sequence results in massive neurodegeneration of the basal ganglia and cortex of patients over the age of 30 (Vonsattel and DiFiglia, 1998; Ross and Tabrizi, 2011; Ross et al., 2014). The role of P2X7 receptor in HD has been still poorly investigated. At the moment, the only study is published by Diaz and collaborators, who by using two distinct mouse models for HD, Tet/HD94 and R6/1 demonstrated that P2X7 receptor expression is increased in HD, and that the receptor channel possesses augmented Ca^{2+} permeability (Díaz-Hernández et al., 2009) (**Figure 3**). The inhibition of the receptor with BBG mitigated motor coordination deficits, cachexia and decreases neuronal loss.

Moreover, *in vitro* analysis revealed that neurons expressing mutant Htt were more sensitive to apoptosis under P2X7 receptor stimulation (Díaz-Hernández et al., 2009). Thus, P2X7 receptors expressed in microglia can promote excitotoxicity in neural cells by inducing glutamate release (Matute, 2012).

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is one of the most prevalent neuromotor diseases in adulthood. The disease is characterized by the death of motoneurons in the motor cortex, brainstem and spinal cord, resulting in muscle impairment and paralysis

(Hardiman et al., 2017). Among the mechanisms involved in neuronal death, neuroinflammation is one of the most established factors. ALS patients present alterations in levels of a range of pro-inflammatory cytokines in the cerebrospinal fluid (Mitchell et al., 2009; Moreno-Martinez et al., 2019), as well as increased rates of reactive cerebral microglial cells (Turner et al., 2004). Depending on the stage of the disease, reactive microglia with protective or cytotoxic properties is found, demonstrating the complexity of neuroinflammation in this disorder (Evans et al., 2013). In this sense, studies relating the P2X7 receptor with ALS show a delicate regulation depending on different factors.

Several studies have been conducted with superoxide dismutase 1 transgenic mice harboring the G92A mutation [SOD1 (G93A)], a well-known ALS model. In this model, onset, progression, and animal survival depend on the mouse gender. Cervetto et al. (2013) showed that inhibition of the P2X7 receptor by BBG at a dose of 45 mg/kg slowed down disease progression in males, but not in females (Cervetto et al., 2013).

In addition, Apolloni et al. (2013a) demonstrated that female SOD1 (G93A) mice with the KO of the P2X7 receptor gene showed increased survival but anticipated the onset of the disease and intensified its progression in males and females. Further, increased astrogliosis and microgliosis and augmented motoneuron death were observed, accompanied by increased pro-inflammatory cytokine production (Apolloni et al., 2013a). Authors used Pfizer KO mice, known to express P2X7 13B and 13C receptors in the brain, which present lower membrane migration and channel function when compared to P2X7A receptors (Masin et al., 2012).

The beneficial effects of P2X7 receptor blockade in ALS supposedly did not depend only on the studied gender, but also on the stage of the disease. In the ALS pre-onset phase in SOD1(G93A) mice, Bartlett et al. (2017) used BBG at a dose of 45 mg/kg, three times a week. They reported that this treatment increased female survival without ameliorating motor performance (Bartlett et al., 2017).

Corroborating these results, treatment of late-pre-onset SOD1 (G93A) mice with BBG at 50 mg/kg, three times a week, delayed disease onset and improved motor performance (Apolloni et al., 2014). In addition, this treatment increased motoneurons survival and decreased microgliosis and expression of pro-inflammatory markers. However, when treated in the onset phase, no neuroprotective effect was observed by P2X7 receptor antagonism. On the other hand, P2X7 receptor activation exerted a protective effect on skeletal muscles of SOD1 (G93A) mice (Fabbrizio et al., 2019). Pre-late-onset treatment with Bz-ATP at a dose of 1 mg/kg for 7 days (i.p.) prevented denervation atrophy of the skeletal muscle. The neuroprotective effect of Bz-ATP could be attributed to another purinergic receptor since this compound is not a selective agonist of P2X7 receptors. Despite that, the P2X7 receptor is known to control proliferation, differentiation, and regeneration in healthy skeletal muscle (**Figure 3**).

In vitro, the co-culture of astrocytes and motoneurons from SOD1 (G93A) mice showed P2X7 receptor involvement in astrocyte activity. The addition of Bz-ATP and ATP induced motoneuron death by astrocytic neurotoxicity. When BBG or apyrase (that increases ATP metabolism and decreases

its concentration) was used, inhibition of neuron death was observed (Gandelman et al., 2010). Although BBG treatment also inhibits P2X4 receptors, activation of these receptors appears to protect motor neurons *in vitro* (Cieślak et al., 2019), indicating that the P2X7 receptor subtype is more likely to be activated in the detrimental effect of Bz-ATP. Subsequently, BBG treatment of motoneurons isolated from rat embryonic spinal cord prevented Bz-ATP-induced cell death. In addition, although low concentrations of ATP induced neuronal death, high concentrations of ATP in the cellular media exerted a protective effect, possibly due to its hydrolysis in ADP and the adenosine-induced activation of P1 receptors. ATP and Bz-ATP induced apoptosis by peroxynitrite production, p38 activation and stimulation of the FAS autocrine signaling pathway (Gandelman et al., 2013).

In vitro studies also corroborate microglial participation in disease development in SOD1 (G93A) mice. Using isolated microglia from these animals, Apolloni et al. (2013b) demonstrated that Bz-ATP increased ROS production and activation of the ERK1/2 signaling pathway (**Figure 3**). The pro-inflammatory effects were alleviated following BBG application. Similar results were obtained in SOD1 (G93A) P2X7 receptor KO microglial cells, strengthening the concept of anti-inflammatory effects promoted by P2X7 receptor antagonism (Apolloni et al., 2013a). Besides inducing pro-inflammatory effects, activation of P2X7 receptors in microglia cells isolated from SOD1 (G93A) mice supposedly also modulate autophagy processes. Bz-ATP increased expression of autophagy markers by inhibiting mTOR phosphorylation. This effect was attenuated by treatment with the P2X7 receptor antagonist A-804598 (Fabbrizio et al., 2017).

Finally, peripheral blood mononuclear cells of patients with ALS showed decreased P2X7 receptor expression. Repeated application of ATP to these cells resulted in diminished intracellular calcium transients compared to controls, demonstrating that decreased P2X7 receptor expression induced dysregulation of intracellular calcium homeostasis (Liu et al., 2016).

In conclusion, P2X7 receptor inhibition supposedly promotes dual effects along the course of ALS. Its effects seem to depend on the time window in which the inhibition started. P2X7 receptor ablation before ALS development in mice seems to be detrimental (Apolloni et al., 2013a). In the asymptomatic phase, P2X7 receptor inhibition did not alter disease onset and survival, although it decreases M1 microglial marker expression (Apolloni et al., 2014). In the pre-onset phase, treatment with BBG increased mice's survival but did not alleviate motor symptoms (Bartlett et al., 2017). When administered at the late pre-onset phase, BBG reduced M1 microglial phenotype and increased anti-inflammatory M2 phenotype along with delayed disease onset and decreased motor symptoms (Apolloni et al., 2014). BBG is known to also inhibit P2X4 receptors to a lesser extent, but the role of P2X4 receptors in the ALS development depends on the cell type. While P2X4 receptor inhibition in microglia cells induces the phenotypic change to M1 microglial cells and promotes inflammation, P2X4 receptor activation appears to protect motor neurons against kainate-induced excitotoxicity *in vitro* (Di Virgilio and Sarti, 2018; Cieślak et al., 2019). Since

BBG treatment induced a decrease in microglial M1 markers, it is more likely that the neuroprotective effects of BBG treatment involves P2X7 receptor inhibition rather than P2X4 receptor inhibition in ALS.

Multiple Sclerosis

Multiple sclerosis is an autoimmune disease with unknown etiology. It is characterized by chronic inflammation with astrogliosis and microgliosis, death of oligodendrocytes, axonal demyelination and subsequent neuronal transmission impairment. Available drugs alleviate symptoms; however, there is no known cure for this disease (Goldenberg, 2012). Sustained activation of the P2X7 receptor is known to induce oligodendrocyte death and demyelination and neuroinflammatory processes and neurodegeneration, which are characteristic for MS. Thus, studies unraveling functions of this receptor in MS development were conducted.

An animal model of autoimmune encephalomyelitis (EAE) is the gold-standard tool for *in vivo* studies, presenting similar features of MS (Lassmann, 1983). In EAE animals, injection of 10 mM BBG into the optic nerve reduced ATP and Bz-ATP-induced demyelination, suggesting that P2X7 receptor activation induced oligodendrocyte excitotoxicity (Matute et al., 2007). BBG also inhibit P2X4 receptors, but their activation in microglia cells is proposed to trigger remyelination process in EAE mice (Di Virgilio and Sarti, 2018), indicating that P2X7 receptor antagonism could be the responsible for BBG treatment protective effects. P2X7 receptor expression during EAE development in rodents has been demonstrated. In the asymptomatic phase of the disease, overexpression of the receptor in astrocytes was observed. At the peak of the characteristic symptoms of the disease, receptor overexpression occurred not only in astrocytes but also in neuronal terminals (Grygorowicz et al., 2010). Following recovery from the disease, the animals showed P2X7 receptor overexpression in glial cells, whose GFAP labeling was increased in the symptomatic phase without reduction after recovery (Grygorowicz et al., 2011) (**Figure 3**). These results were later confirmed, in which reactive astrocytes in the early phase of the disease expressed P2X7 receptors. Treatment with BBG (50 mg/kg) for 6 days alleviated the appearance of the characteristic symptoms of the EAE rat model, accompanied by reduction in reactive astrocyte labeling (Grygorowicz et al., 2016). Microglial cell analysis also yielded interesting results. In the asymptomatic phase of EAE, microglial cells showed P2X7 expression in active and resting phenotypes, and treatment with 50 mg/kg BBG for 6 days reduced microglial activation and pro-inflammatory cytokine release (Grygorowicz and Strużyńska, 2019).

In the Pfizer P2X7 receptor KO animals, induction of the EAE model resulted in a more severe pathological scenario of the disease. Moreover the authors of this study (Chen and Brosnan, 2006) injected bone marrow cells from P2X7 receptor KO mice into wild-type animals and detected a greater susceptibility to the disease. *In vitro* co-culture of P2X7 receptor KO macrophages and lymphocytes revealed increased lymphocyte proliferation together with decreased apoptotic activity. These results suggest that enhanced disease susceptibility of P2X7 receptor KO

animals may be due to decreased lymphocyte apoptosis rates (Chen and Brosnan, 2006). Controversially, Sharp and colleagues showed that GlaxoSmithKline P2X7 receptor KO mice presented four times less development of the EAE model, with reduced astrocyte activation and axonal damage. On the other hand, they detected an increase in pro-inflammatory cytokine production in splenic T-cells (Sharp et al., 2008), explained by expression of P2X7K receptors in these cells (Bartlett et al., 2014). Although controversial, these results ensure that P2X7 receptors play an important role in the development of the EAE model, both peripherally and in the central nervous system.

Activation of P2X7 receptors is known to induce opening of pannexin-1 associated membrane pores, with increased release of ATP. In this sense, pannexin-1 KO mice showed a decrease in EAE onset rates, accompanied by diminished mortality. In addition, ATP release in the spinal cord was diminished, accompanied by an increase in P2X7 receptor expression. In the long term, these animals developed symptoms as severely as wild-type animals did when submitted to the EAE model. The authors of the work (Lutz et al., 2013) suggested that increased P2X7 receptor expression is a mechanism to counteract the decrease in ATP release due to the absence of pannexin-1, and that this mechanism may be the reason for the similar development of symptoms. When treated with the pannexin-1 inhibitor mefloquine wild type EAE animals showed less severity in EAE development (Lutz et al., 2013).

The P2X7 receptor is associated with reactive microglia, as shown for microglial cells extracted during the autopsy of individuals with MS (Beaino et al., 2017). In addition, P2X7 receptor activation may play a role in the upregulation of IL-1β through nitric oxide synthase expression (Narcisse et al., 2005). P2X7 receptor expression was detected in reactive astrocytes in postmortem brains, showing expression upregulation in the parenchyma of the frontal cortex and in microglial cells from spinal cord and white brain matter (Narcisse et al., 2005; Yiangou et al., 2006; Amadio et al., 2017). P2X7 receptor expression was reduced in peripheral blood mononuclear cells (PBMCs) during acute disease phase, possibly due to autocrine and paracrine mechanisms resulting from inflammatory processes. The obtained results indicate that P2X7 receptor expression downregulation in monocytes and upregulation of expression in astrocytes participate at the inflammatory process of MS (Amadio et al., 2017). In contrast, PBMCs from MS patients had no difference in P2X7 receptor expression when compared to healthy individuals (Caragnano et al., 2012). However, when treated with glatiramer acetate, a compound used for MS treatment, P2X7 receptor and CD39 expression rates were reduced in PBMCs. These data were corroborated by in vitro studies of PBMCs, which when treated with glatiramer acetate showed a decrease in P2X7 receptor expression and a tendency to reduced IL-1β and increased CD39 expression (Caragnano et al., 2012).

Besides rare mutations in the P2X7 receptor gene found in familial MS (Sadovnick et al., 2017; Zrzavy et al., 2019), patients with mutations of Arg307Gln (rs28360457), which cause a substantial loss in membrane pore formation, are up to twice less frequent in MS patients, indicating a protective effect of

this mutation (Gu et al., 2015). The opposite occurs when the mutation involves a P2X7 receptor gain-of-function that increases receptor channel permeability for Ca^{2+} such as the Ala76Val polymorphism, which is more common in MS patients (Oyanguren-Desez et al., 2011) (**Figure 2**).

Altogether, in vivo and in vitro evidence in animal models and patient samples indicates that the P2X7 receptor is closely related to MS pathology. Its expression is increased in microglia and reactive astrocytes resulting from inflammatory processes, and interventions that downregulate expression or activity of this receptor have neuroprotective effects. Moreover, although several studies used BBG as antagonist for P2X7 receptors, and this compound also inhibits P2X4 receptors, activation of the latter is known to induce microglial changes towards the M2 phenotype exerting remyelination effects in EAE mice (Di Virgilio and Sarti, 2018). Additionally, outcomes of P2X7 receptor ablation before EAE development are not clear, since different P2X7 receptor KO mice present different outcomes.

P2X7 RECEPTOR ROLES IN PSYCHIATRIC DISORDERS

As reviewed by Cheffer et al. (2018), a range of purinergic receptors are involved in psychiatric disorders. As discussed below, the P2X7 receptor also seems to influence development, vulnerability and severity of these disorders.

Depressive Disorders

Major depressive disorder (MDD) is estimated to affect about 322 million people worldwide, which represents 4.4% of the global population (World Health Organization, 2017). Prevalence rates vary by sex (5.1% of females and 3.6% of males) and by age (peaking in the older adulthood, between 55 and 74 years old) (World Health Organization, 2017). As described by several studies, MDD has a high social and economic impact (Wang et al., 2003; Greenberg et al., 2015), which could be attenuated by more appropriated treatments (Chisholm et al., 2016). However, about 65% of patients with MDD fail to achieve remission and about 33% do not respond to the treatment initially prescribed (Schatzberg, 1999; Trivedi et al., 2008). A possible explanation for the ineffectiveness of antidepressants in some patients is that most of them acts through facilitation of monoaminergic neurotransmission and studies from the last decade show that depression etiology involves more than this system (Kendler et al., 2006; Dean and Keshavan, 2017).

Depressive disorders result from a combination of environmental influence, personality traits, genetic and epigenetic factors leading to neuroendocrine dysfunction (hypothalamic–pituitary–adrenal axis imbalance), neurochemical alterations (impaired monoaminergic neurotransmission, increased glutamate levels and enhanced neuroimmune response) and decreased neuroplasticity (Kendler et al., 2006; Dean and Keshavan, 2017). As recently reviewed by Ribeiro and co-workers the P2X7 receptor is a core regulator of such neurochemical and neuroplastic mechanisms (Ribeiro et al., 2019a). Based on that, it is not surprising that several

studies indicate P2X7 receptor involvement in mood disorders as discussed in the following.

A pioneering work showed an association between the presence of the SNP rs2230912 in the gene coding for P2X7 receptor with MDD development (Lucae et al., 2006). Accordingly, the SNP rs2230912 was also associated with mood disorders, longer depressive episodes (Soronen et al., 2011) and increased severity of the depressive symptoms (Hejjas et al., 2009). However, the case-control study performed by Hejjas et al. (2009) found no differences in the presence of these polymorphisms between patients suffering from MDD and controls. Moreover, opposite results were found by two meta-analysis studies: Feng et al. (2014) reported that there was no association between rs2230912 polymorphism and MDD development; however, Czamara et al. (2018) showed a positive correlation (Feng et al., 2014; Czamara et al., 2018) (**Figure 2**). It is noteworthy that the latter work included more validated studies, which could explain the different results.

In addition, mice expressing either normal human P2X7 receptors (hP2X7 receptor – wild type) or receptors expressed by an altered gene (hP2X7 receptor – rs2230912), did not present any behavioral changes (Metzger et al., 2017b). However, hP2X7 receptor – rs2230912 mice showed increased vulnerability to chronic social defeat stress. These results indicate that heterozygotic individuals may be more susceptible to development of MDD through interactions between genetic predisposition and stress exposure (Metzger et al., 2017b). In accordance with this idea, the gene polymorphism rs7958311 in P2X7 receptor was correlated with MDD development in individuals with previous history of stress exposure (Gonda et al., 2018) (**Figure 2**).

Beyond the evidence provided by human studies, *in vitro* and *in vivo* experiments may also help to understand the role of the P2X7 receptor in depression and in the mechanisms underlying therapeutic and/or side effects induced by antidepressants. For this purpose, the effects of antidepressant treatment on the expression/function of the P2X7 receptor has been investigated. In a whole-cell patch-clamp study, paroxetine, but not fluoxetine nor desipramine administration, reduced the inward currents evoked by Bz-ATP on cloned rat P2X7 receptors expressed in HEK293 cells (Wang et al., 2016). In another study, paroxetine inhibited, while fluoxetine and clomipramine potentiated ATP-induced dye uptake in HEK-293 cells expressing recombinant human P2X7 receptors (Dao-Ung et al., 2015). *In vivo*, antidepressant-like effect induced by clemasine (Su et al., 2018), ketamine (Tan et al., 2017) and imipramine (Ribeiro et al., 2019b) were associated with diminished P2X7 receptor levels in the hippocampus of stressed animals. These results suggest that P2X7 receptor activity/expression can be modulated by different antidepressants, revealing a potential mechanism by which these drugs may induce their therapeutic effects. Accordingly, mice exposed to chronic unpredictable mild stress (CUMS) (Su et al., 2018) or chronic restraint stress (Tan et al., 2017) presented enhanced P2X7 receptor expression in the hippocampus. However, there are also animal studies showing no alterations (Yue et al., 2017) or even a reduction (Kongsui et al., 2014) in hippocampal P2X7 receptor levels induced

by stress exposure. The discrepant data may be explained by different techniques used to determine P2X7 receptor levels (Western blotting versus immunohistochemistry), different stress protocols, or it may indicate a more complex role of P2X7 receptor in stress induced consequences (**Figure 3**).

Aiming to better understand P2X7 receptor involvement in stress response, the effects of P2X7 receptor inhibition has been studied. P2X7 receptor KO mice presented antidepressant-related behavior in both forced swim test (FST) and tail suspension test (TST), two experimental approaches to predict antidepressant effects of drugs (Basso et al., 2009; Csölle et al., 2013a,b). In addition, P2X7 receptor KO mice demonstrated improved responses to a sub-effective dose of imipramine in the FST (Basso et al., 2009). Despite these results, Boucher and co-workers observed a decrease in the immobility time of P2X7 receptor KO mice only after repeated exposure to the FST (Boucher et al., 2011). Altogether, data from P2X7 receptor KO mice indicate that P2X7 receptor absence results in increased resilience to stress, and a phenotype showing antidepressant-related behaviors.

Pharmacological studies in rodents using antagonists with different affinities for P2X7 receptor further support this hypothesis. Pereira and co-workers observed that acute treatment with PPADS (12.5 mg/kg), a pan antagonist for P2 receptors, or iso-PPADS (12.5 or 25 mg/kg), an antagonist of P2X receptors, decreased the immobility time in the FST (Pereira et al., 2013). Csölle et al. (2013b) observed that systemic administration of BBG at dose of 50 mg/kg/day during 4 days, increased sucrose consumption and decreased the immobility time in the TST of mice pretreated with LPS. In another study from the same research group subchronic (7 days) but not acute treatment with BBG (50 mg/kg/day) decreased the immobility time of mice exposed to TST (Csölle et al., 2013a). Mice systemically treated with BBG (50 mg/kg/day) during 8 weeks (Farooq et al., 2018) or rats treated with A-804598, at a dose of 5 mg/kg twice daily for 4 weeks (Iwata et al., 2016), reversed behavioral alterations induced by CUMS exposure. In accordance with these data, 7 days of treatment with BBG (50 mg/kg/day) decreased the number of escape failures induced by inescapable foot shocks application (Ribeiro et al., 2019b). Additionally, 7 days of treatment with A-804598 (30 mg/kg/day) induced antidepressant-like effects in the flinders sensitive line rats, an animal model of depression based on selective breeding (Ribeiro et al., 2019c). Intracerebral administration of P2X7 receptor antagonists have been also carried out in order to investigate the role of these receptors in specific brain regions. Interestingly, microinjection of P2X7 receptor antagonists (BBG or A-438079) into the rat hippocampus during 3 weeks prevented the development of depression-related behaviors induced by CUMS exposure, while the administration of P2 receptors agonists (ATP or Bz-ATP) for the same period caused depressive-like behaviors similar to those observed after stress exposure (Yue et al., 2017).

Altogether, pharmacological and genetic findings indicate that P2X7 receptor inhibition induces antidepressant-related effects in animals. This response may be mainly associated with the blockade of P2X7 receptors expressed in the hippocampus,

although the involvement of other brain structures needs to be further investigated. Regardless the region responsible for the effects induced by systemic administration of P2X7 receptor antagonists, the behavioral response points this receptor as a possible target for depression therapy.

Bipolar Disorder

Bipolar disorder is an incapacitating, chronic and severe mental disorder that occurs in a cyclic course. Patients with bipolar I disorder (BDI) present an exacerbated mood elevation, mania episodes and usually experience major depression. Bipolar II patients (BDII) exhibit an elevation of mood, named hypomania, and a history of major depression without mania episodes. The whole spectrum of BD is prevalent in approximately 2.4% of population, whereas the prevalence of BDI and BDII are 0.6 and 0.4%, respectively (Merikangas et al., 2011). There is several evidence that BD may progress and present neurodegenerative components, once patients exhibit symptoms worsening, gradual cognitive impairment and brain atrophy (Rao et al., 2010).

The neurobiological processes of BD remain poorly understood. The pathways most associated hitherto include monoaminergic neurotransmission, such as dopaminergic, serotonergic, and noradrenergic systems (Grande et al., 2016), redox imbalance (Versace et al., 2014) and neuroinflammation. Some contradictory results exist regarding the neuroinflammation state in BD. BD is a highly heterogeneous disorder and the classification, cycling phase, number of episodes, and medication can vary widely among patients, which can implicate different inflammatory cytokine patterns present in BD patients. Using a meta-analytic approach, serum or plasma samples evidenced highly concentrated soluble IL-2 receptor, TNF-a, soluble TNF receptor type 1, soluble IL-6, and IL-4 in bipolar patients. Overall, there were not any differences between other analyzed anti-inflammatory and pro-inflammatory cytokines (Munkholm et al., 2013).

Bipolar disorder is extremely difficult to model in rodents since the mechanism behind the maniac and depressive cycle is not well established. Thus, animal models are employed that mimic the state of mania. A mouse strain that naturally presents a mania-like phenotype showed downregulation of P2X7 receptor expression (Saul et al., 2012). In contrast, genetic deletion of P2X7 receptor protected the abnormal locomotor activity by acute amphetamine administration (Csölle et al., 2013b; Gubert et al., 2016). In the mania animal model induced by chronic administration of amphetamine, pharmacological antagonism with A-438079 and genetic deletion of P2X7 receptor completely reverted increased locomotor activity induced by amphetamine (Gubert et al., 2016). Additionally, A-438079 abolished the release of pro-inflammatory cytokines IL-1β and TNF-α and lipid peroxidation in hippocampus (Gubert et al., 2016). Using the same animal model, BBG treatment prevented hyperlocomotion, DOPAC augmentation in the hippocampus, increased NTPDase3 expression and astrogliosis induced by amphetamine (Gubert et al., 2019b) (**Figure 3**). Although in the last work only the non-specific antagonist BBG was used, Gubert et al. (2016) found similar results when BBG or the specific antagonist A-438079 were administrated. These studies evidence a reproducibility

in P2X7 receptor antagonism in the mania model induced by amphetamine, strengthening the possible role of P2X7 receptor in mania-like state in BD.

There are several studies of genetic associations between P2X7 receptor polymorphisms and BD development. However, inconsistent findings made the identification of any association impossible. The rs2230912 is a SNP in the P2X7 receptor gene that promotes gain of function and was previously associated with increased risk of BD development in patients from the United Kingdom and Ireland (McQuillin et al., 2009) and Canada (Barden et al., 2006). Further, BD patients that presented rs2230912 and rs208294 polymorphisms spent more time in the symptomatic stage than patients without these alleles (Soronen et al., 2011). Nevertheless, this finding was not appropriately replicated in other populations studies. A multi-centric analysis conducted in individuals from Germany, Poland, Romania, and Russia evidenced no allelic or genotypic association between rs2230912 and BDI (Grigoroiu-Serbanescu et al., 2009). Studies in Swedish BD patients revealed an association between rs1718119 and rs1621388 polymorphisms and cognitive features of mania – distractibility, thought disorder, and talkativeness. Still, the rs2230912 polymorphism presented no association with BD (Backlund et al., 2011). A study that analyzed nine variants of P2X7 receptor polymorphisms, such as rs591874, rs208293, rs1186055, rs208298, rs503720, rs1718133, rs1718119, rs2230912, and rs1621388, in United Kingdom individuals found that these polymorphisms did not have any effects on BDI susceptibility (Green et al., 2009). A recent study conducted in Brazilian patients evidenced a decrease in 1513C allele frequency and a potential increase in 1513A A/AC genotype frequency of rs3751143 polymorphism in BD patients (Gubert et al., 2019a) (**Figure 2**). All these polymorphisms in the P2X7 receptor gene represent a gain of function, which could indicate potential influence of the P2X7 receptor behind the genetic predisposal of BD development.

Schizophrenia

Schizophrenia (SCZ) is a complex, multifactorial, heterogeneous, and severe psychiatric disorder. SCZ symptomatology is classified by three major categories: (1) positive symptoms, in which the patient may present disturbance of thinking, delusions and hallucinations, named psychotic symptoms; (2) negative symptoms that are characterized by impaired motivation, decrease in spontaneous speech, and social withdrawal; and (3) cognitive symptoms, which the core features may present impairments in working memory, attention, problem-solving, and executive functioning (van Os and Kapur, 2009). Many efforts have been placed to understand the molecular mechanisms that cause SCZ, however, the full complexity of this disorder remains unknown. SCZ is a highly polygenic (Owen et al., 2016) and many environmental factors have been already associated (Byrne et al., 2004; Allardyce and Boydell, 2006; Varese et al., 2012; Cantor-Graae and Pedersen, 2013; Moustafa et al., 2017). Besides, it is already known that SCZ is a neurodevelopmental disorder and maternal complications may be risk factors (Khashan et al., 2008; Brown, 2011, 2012; Khandaker et al., 2013). There are multiple lines

of evidence supporting the impaired function in dopamine, glutamate and GABA neurotransmission (Schwartz et al., 2012). Similarly, several neurochemical dysfunctions are stated in the kynurenine pathway (Kindler et al., 2019), redox dysregulation (Do et al., 2015), and neuroinflammation (Na et al., 2014; Marques et al., 2019).

Few clinical data are available regarding P2X7 receptor participation in SCZ pathophysiology. Two antipsychotics drugs, prochlorperazine and trifluoperazine, may inhibit human P2X7 receptor function (Hempel et al., 2013). Further, prochlorperazine, a drug with strong antipsychotic action, could act as a negative allosteric modulator of P2X7 receptor activity (Hempel et al., 2013). A populational study conducted with SCZ patients from Denmark analyzed nine SNPs of the P2X7 receptor – rs28360447, rs208294, rs28360457, rs1718119, rs2230911, rs2230912, rs3751143, rs1653624, and rs35933842 – and did not observe any associations between SCZ and these polymorphisms of P2X7 receptor (Hansen et al., 2008) (**Figure 2**).

It is a tremendous challenge to mimic SCZ using animal models due to its high complexity, multifactorial component, and difficulty to distinguish and analyze positive symptoms of these disorders. Phencyclidine (PCP) is a compound largely used as an inductor for animal models of SCZ once the rodents present some similar features in their behavior. In the acute PCP mouse model, the pharmacological blockade with JNJ-47965567 and genetic deletion of the P2X7 receptor alleviated some behavioral parameters and also alteration of gene expression of GABA receptor subunits and neuregulin 1 in the prefrontal cortex (Koványi et al., 2016). Overall, there is lack of evidence supporting the role of the P2X7 receptor in the neurobiology of SCZ. It is a poorly explored field and more studies are needed to indicate whether or not there is association.

Anxiety

Anxiety disorders belong to the most prevalent and disabling psychiatric disorders, substantially impacting life quality. It is estimated that 25% of the population will suffer at least one episode of this disease in adulthood. Types of anxiety disorders include separation anxiety disorder, specific phobia, social anxiety disorder, panic disorder, agoraphobia, generalized anxiety disorder, and drug-induced anxiety disorder. Symptoms include anxiety, excessive fear and other mood disturbances (Kessler et al., 2005). Anxiety disorders are often accompanied by other psychiatric disorders, such as MDD and BD (Schaffer et al., 2012).

Current treatments include serotonin and norepinephrine reuptake inhibitors, benzodiazepines and antidepressant drugs. However, these are partially efficient according to patient histories and the type of anxiety disorder (Murrough et al., 2015). Thus, the identification of specific targets for novel therapeutic approaches is urgent.

In PBMCs from patients with anxiety and depression, an increase in P2X7 receptor expression was found after ATP stimulation. In the same cells, patients with comorbidity of anxiety and Sjogren's syndrome have higher P2X7 receptor expression when compared to control healthy individuals (Xie et al., 2014).

Several studies show the relationship between the chromosome 12q2431, in which the P2X7 receptor gene is inserted, and the development of mood disorders. Thus, polymorphisms of this receptor are widely studied in mood disorders. The SNP rs1718119 with the Thr348Ala mutation was not related to anxiety onset in patients (Erhardt et al., 2007). Although the P2X7 receptor rs2230912 Gln460Arg polymorphism did not present any relation to mood disorders in case-control analysis, this receptor induces higher symptomatic severity scale scores of patients with G-allele (Nagy et al., 2008; Hejjas et al., 2009). In a cohort study, this same SNP was associated with a higher risk of developing mood disorders and alcoholism, including anxiety (Soronen et al., 2011). This study also identified the rs208294 His155Tyr polymorphism as a possible risk factor for disease development (Soronen et al., 2011). In addition, the P2X7 receptor variant rs208294 has been associated with neuroticism-mediated outcomes of mood disorder, a personality trait that indicates vulnerability to the onset of anxiety in stressful situations (Mantere et al., 2012) (**Figure 2**).

P2X7 receptor KO mice show controversial results regarding anxiety-like behavior. Despite showing decreased depressive behavior, Pfizer P2X7 receptor KO animals showed no anxiolytic effect in the elevated plus maze test (Basso et al., 2009). In contrast, Boucher et al. (2011) found anxiety-like behavior in this same test, but not in the light dark emergence test (Boucher et al., 2011). P2X7 receptor KO mice also exhibited anxiety-like behavior in the elevated plus maze test when subjected to contextual fear condition (Domingos et al., 2018).

The P2X7 receptor also presented discrepant results regarding its involvement in inducing anxiety-like behavior in different animal models. Inhibition of the P2X7 receptor with A-438079 (10 mg/kg) augmented anxiety-like behavior of mice subjected to the contextual fear condition model (Domingos et al., 2018). Antagonism using intraperitoneal injections of A804598 for 25 days decreased this behavior in mice subjected to high fat diet (Dutheil et al., 2016), possibly by blocking the formation of inflammasomes. However, this same compound had also an anxiolytic effect in an unpredictable chronic stress model, blocking the release of IL-1β, TNF-α and inflammasome formation (Iwata et al., 2016).

Overall, effects of P2X7 receptor activity modulation on animal anxiety parameters has yet to be elucidated. Ablation of P2X7 receptor expression did not prevent the onset of symptoms, and receptor antagonism induce pro- and anti-anxiety effects in different animal models.

BRAIN TUMORS

Brain tumors are intracranial neoplasms that account for 2% of all cancers (Gould, 2018), while being the second most common cancer among 0 to 14-year-old children. Surpassing even leukemia, brain cancers are the leading cause of oncologic death in this age group (American Brain Tumor Association, 2019). Importantly, the brain is a very fertile soil for metastatic seeding, so that brain metastases incidence is estimated to be at

least 10 times higher than that of primary brain tumors (Vargo, 2017). In fact, 30% of all people with cancers in other body parts will present brain metastases (Gould, 2018). Among primary malignant brain tumors, 80% of all cases are gliomas, malignant tumors raising from glial cells (Gould, 2018).

Although prognosis greatly varies, the incidence of near- and long-term disabilities is notably high (Mukand et al., 2001). Both the tumor itself and the frequently associated perilesional edema, which can reach a several-fold greater volume than the tumor itself, account for the functional neurological consequences (Tran et al., 2019). Indeed, brain tumors cause severe economic impacts not only due to direct treatment and rehabilitation costs, but also due to productivity loss (Su and Abdullah, 2016).

Among candidate molecular targets for anti-cancer drug development, the P2X7 receptor has received great attention. In fact, high ATP levels are a common feature in the tumor microenvironment, reaching concentrations of up to hundreds of micromolar (Pellegatti et al., 2008), a range of concentration capable of activating P2X7 receptors (North and Barnard, 1997). Thus, it is not surprising that P2X7 receptors emerge as central players of purinergic signaling in the tumor microenvironment. In agreement, P2X7 receptor expression is upregulated in several tumor types (Adinolfi et al., 2002; Slater et al., 2004; Solini et al., 2008; Ryu et al., 2011; Arnaud-Sampaio et al., 2019). Glioma cell lines of human (U-138MG, U-251MG, M059J) (Gehring et al., 2012), rat (C6) (Wei et al., 2008), and mouse (GL261) (Tamajusuku et al., 2010) origin express P2X7 receptors as well. Importantly, glioma cells have decreased sensitivity to the cytotoxic effects of extracellular ATP in comparison to healthy tissue cells (Morrone et al., 2005), and glioma cells show less ATP hydrolysis (Wink et al., 2003), favoring the maintenance of high extracellular ATP concentrations. Furthermore, stimulation by extracellular ATP drives the release of glutamate by GL261 glioma cells, an effect partially reversed by P2X7 receptor antagonism (Strong et al., 2018). Elevated levels of both ATP and glutamate mediate cytotoxic effects on the boundaries of the tumor, favoring its expansion and growth (de Groot and Sontheimer, 2011; Strong et al., 2018).

Brain tumor microenvironment is composed by tumor and stromal cells, as reactive astrocytes, fibroblasts and myeloid-derived cells, including microglia (Volak et al., 2018). Therefore, P2X7 receptor expression in the tumor mass may occur in different cell types, leading to particular downstream responses, which may be pro- or anti-tumoral depending on the context. The analysis of human glioma samples revealed that microglial cells confined within the tumor had increased P2X7 receptor expression, and pharmacological inhibition of the receptor significantly decreased the number of glioma cells (Monif et al., 2014).

In a brain tumor model established by intrastriatal injection of C6 glioma cells in rats, pharmacological inhibition of P2X7 receptor by BBG decreased tumor growth. In vitro, BBG treatment decreased the receptor expression and prevented chemotaxis induced by Bz-ATP (Ryu et al., 2011), pointing to a pro-tumoral intrinsic activity of P2X7 receptor in this model. In agreement, stimulation of human glioma cells with Bz-ATP increased cell proliferation and migration, an effect counteracted

by an inhibitor of the MEK/ERK pathway, implicating this pathway in P2X7 receptor-mediated proliferative effects (Ji et al., 2018) (**Figure 3**). Consistently, overexpression of the P2X7 receptor in a naturally low-expressing human glioma cell line conferred modest in vitro growth advantages, but largely accelerated tumor growth in vivo (Bergamin et al., 2019), reinforcing a trophic role for this receptor. Also, in a mouse model of neuroblastoma, a rare intracranial tumor that affects immature or developing cells of the nervous system, chronic blockade of the P2X7 receptor in tumor-bearing mice diminished progression and metastasis (Ulrich et al., 2018).

In contrast, another study found that P2X7 receptor blockade by BBG increased C6 glioma cell proliferation, an effect corroborated by enhanced tumor growth observed in rats that received intracranial transplantation of C6 glioma cells either due to p2rx7 gene knockdown or pharmacological P2X7 receptor blockade (Fang et al., 2013). Conflicting findings were attributed by the authors to different periods and doses of BBG treatment, which would lead to distinct microglial responses.

When expressed both in glioma cells and in glioma-infiltrating microglia, the P2X7 receptor mediates the release of pro-inflammatory factors, as monocyte inflammatory protein 1α (MIP-1α) (Fang et al., 2011), monocyte chemoattractant protein 1 (MCP-1) (Wei et al., 2008; Fang et al., 2011; Braganhol et al., 2015), IL-8 (Wei et al., 2008; Braganhol et al., 2015) and VEGF (Wei et al., 2008). In fact, P2X7 receptor expression in tumor bearing-hosts is essential for mounting an effective anti-tumoral immune response, so that genetic deletion or pharmacological blockade of the receptor increased the incidence of tumors in a murine colitis-associated cancer model (Hofman et al., 2015). Furthermore, P2X7 receptor-deficient tumor-bearing mice undergo a shift toward an immunosuppressive response (De Marchi et al., 2019) and show accelerated tumor progression (Adinolfi et al., 2015).

A comparison between human glioma cell lines showed that those with upregulated P2X7 receptor expression exhibited higher sensitivity to irradiation (Gehring et al., 2012). Further studies corroborated that the P2X7 receptor acts synergistically with radiotherapy promoting cytotoxicity, and the level of P2X7 receptor expression is a good prognosis predictor for radiotherapy response in gliomas (Gehring et al., 2015). Treatments with high ATP and Bz-ATP concentrations also potentialized in vitro cytotoxic effects of temozolomide, a drug of choice for glioblastoma treatment, in human glioblastoma cells (D'Alimonte et al., 2015). In agreement, the P2X7 receptor is implicated in the ATP-induced necrotic death of glioblastoma murine cells, supporting its role in killing tumoral cells (Tamajusuku et al., 2010), despite the evidence of glioma resistance to ATP-induced cytotoxicity (Morrone et al., 2005).

In summary, responses triggered by P2X7 receptor highly depend on the expression levels of the receptor, on the stimulation tonus and on the cell type, and the context of tumor microenvironment seems crucial for determining whether P2X7 receptor activation will end up being pro- or anti-tumorigenic. Ultimately, translating existing evidence into therapeutically useful approaches demands a fine

resolution between the distinct phenomena mediated by P2X7 receptors. Adopting optimized experimental designs is crucial to move forward, highlighting how and when P2X7 receptor actions are relevant for tumoral pathophysiology. Experimental design should take into account the complexity of the tumor microenvironment, the different stages of tumor development and the numerous existing splicing variants of the P2X7 receptor gene. Furthermore, findings should combine multiple strategies and rely on both gene expression modulation tools and specific agonists and antagonists, so that conclusions are reproducible and robust. In fact, a considerable part of the available evidence relies on pharmacological modulators that could target other purinergic receptors, as previously mentioned. IC_{50} values for inhibition of other purinergic receptors by BBG fall in the micromolar range, and experimental concentrations for P2X7 receptor inhibition are traditionally limited to hundreds of nanomolar. However, especially in human cells, in which IC_{50} values for P2X7 receptor and P2X4 receptor inhibition differ by only approximately an order of magnitude, much closer than those observed, i.e., in rats (Jiang et al., 2000), overlapping inhibition of both receptors may occur. In spite of that, evidence implicating P2X4 receptor functions in tumor biology is scarce, and mostly related to its inflammatory roles (Guo et al., 2004). In fact, gliomas poorly express P2X4 receptors, and its presence has no prognostic value (The Human Protein Atlas, 2020).

BRAIN-PENETRANT P2X7 RECEPTOR ANTAGONISTS

As discussed so far, P2X7 receptor blockade may be a viable approach for treating brain diseases. Although a range of P2X7 receptor antagonists were developed, some of them are not capable of passing the blood-brain barrier (**Table 1**).

Compounds produced by GlaxoSmithKline (GSK-1482160) and Janssen (JNJ-54175446 and JNJ-55308942) were the first to present both effects in rodents and CNS permeability (Letavic et al., 2017; Territo et al., 2017; Chrovian et al., 2018). The observed *in vivo* activity stimulated the use of target engagement assays to drive development of new drugs, as well as allowed pharmacological tests in rodent models of diseases (Bhattacharya, 2018). In this way, GSK and Janssen advanced in developing other P2X7 receptor antagonists capable of penetrating the blood-brain barrier: GSK compound 16 (Beswick et al., 2010), JNJ-42253432 (Letavic et al., 2013; Lord et al., 2014), JNJ-47965567 (Bhattacharya et al., 2013; Letavic et al., 2013), and JNJ-54166060 (Swanson et al., 2016). In addition, Abbott Laboratories synthesized brain-penetrant P2X7 receptor antagonists, namely: A-438079 (Nelson et al., 2006), A-740003 (Honore et al., 2006), A-804598 (Donnelly-Roberts et al., 2009; Able et al., 2011), and A-839977 (Honore et al., 2009).

Despite the development of several compounds [for detailed reviews see Rech et al. (2016), Pevarello et al. (2017)], the only CNS-permeable P2X7 receptor antagonist that advanced to clinical trials was GSK-1482160. Besides promising initial data, the GSK-1482160 did not present the safety margins to achieve such sustained inhibition, and consequently its development was terminated (Ali et al., 2013).

Currently, Affectis Pharmaceuticals disclosed the use of the brain-penetrant P2X7 antagonist AFC-5128 for neuropathic pain and MS treatment, as stated at the company's website[1]. Moreover, Alzheimer's Drug Discovery Foundation has been supporting Axxam to identify selective P2X7 receptor antagonists for AD treatment.

CONCLUDING REMARKS

The P2X7 receptor has become a very popular target in the purinergic signaling research. This review collected evidence for P2X7 receptor role in CNS diseases, although further studies are needed for a better understanding of this involvement. The neuroinflammation process is largely prominent in CNS diseases, mainly those covered in this review. It is robustly established that P2X7 receptor activation promotes proinflammatory cytokines release, whereas P2X7 receptor blockade efficiently inhibit the neuroinflammatory process. Additionally, blockade of P2X7 receptor signaling may reduce hippocampal amyloid plaques in AD; regenerate dopaminergic neurons of nigrostriatal pathway in PD; delay the ALS onset, progression, and motor performance; decrease MS-related symptoms and microglial activation in this condition; exhibit anti-depressant properties; reduce features related to mania; and decrease tumor growth. Degeneration of neural cells as presented in these conditions may increase the extracellular ATP levels, leading to overactivation of P2X7 receptors. Furthermore, AD, PD, MS, MDD, and brain tumors present increased P2X7 receptor expression. In view of that, we propose a signal amplification of P2X7 receptors in these diseases.

Pharmacological and genetic studies also contributed to elucidate the neurobiology of these conditions. However, here we provide evidence of the lack specificity of some antagonists and antibodies related to the P2X7 receptor. BBG, for example, is still widely used in the literature due to the low cost and blood-brain barrier permeability despite its non-specificity. Therefore, critical analysis regarding P2X7 receptor studies is extremely necessary.

The most studied SNPs of the P2X7 receptor result in loss or gain-of-function, and several studies associate these SNPs with disease development, symptomatology or disease worsening concerning AD, BD, MS, MDD, PD, and anxiety. Regarding SCZ and anxiety, the role of P2X7 receptor should be further explored to clarify its involvement in the pathogenesis of these disorders. Altogether, the studies presented here show the involvement of the P2X7 receptor in pathologies and the therapeutic potential of inhibiting this receptor in the treatment of brain diseases. Herewith, we suggest that these effects are due to the resolution of neuroinflammation components of the aforementioned diseases.

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[1] http://www.affectis.com/afc5128.html

AUTHOR CONTRIBUTIONS

RA wrote the Schizophrenia, Bipolar Disorder, and Conclusion sections and prepared the **Figures 2, 3** and **Table 1**. ÁO-G wrote the Parkinson's Disease, Amyotrophic Lateral Sclerosis, Multiple Sclerosis, Anxiety, and Conclusion sections and prepared the **Table 1**. DR wrote the Alzheimer's Disease, Depressive Disorders, and Brain Penetrant Drugs sections and prepared the **Table 1**. TG wrote the Introduction and Huntington's Disease sections and prepared the **Figure 1**. VA-S wrote the Brain Tumor section. HU and CL conceptualized, supervised manuscript elaboration, edited, revised, and critically overviewed the manuscript. All authors contributed to the article and approved the submitted version.

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4.2.6. ATP and spontaneous calcium oscillation control neural stem cell fate determination in Huntington's disease: a novel approach for cell clock research. *Molecular Psychiatry*, 2020.

ARTICLE

ATP and spontaneous calcium oscillations control neural stem cell fate determination in Huntington's disease: a novel approach for cell clock research

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Abstract

Calcium, the most versatile second messenger, regulates essential biology including crucial cellular events in embryogenesis. We investigated impacts of calcium channels and purinoceptors on neuronal differentiation of normal mouse embryonic stem cells (ESCs), with outcomes being compared to those of in vitro models of Huntington's disease (HD). Intracellular calcium oscillations tracked via real-time fluorescence and luminescence microscopy revealed a significant correlation between calcium transient activity and rhythmic proneuronal transcription factor expression in ESCs stably expressing ASCL-1 or neurogenin-2 promoters fused to luciferase reporter genes. We uncovered that pharmacological manipulation of L-type voltage-gated calcium channels (VGCCs) and purinoceptors induced a two-step process of neuronal differentiation. Specifically, L-type calcium channel-mediated augmentation of spike-like calcium oscillations first promoted stable expression of ASCL-1 in differentiating ESCs, which following P2Y2 purinoceptor activation matured into GABAergic neurons. By contrast, there was neither spike-like calcium oscillations nor responsive P2Y2 receptors in HD-modeling stem cells in vitro. The data shed new light on mechanisms underlying neurogenesis of inhibitory neurons. Moreover, our approach may be tailored to identify pathogenic triggers of other developmental neurological disorders for devising targeted therapies.

Introduction

The process by which a single-cell zygote becomes a complex multicellular individual is modulated by various biological variables that converge into a perfect regimen of embryonic development [1, 2]. Embryonic stem cells (ESCs) have the intriguing capability of stimulating the in vitro development of the three embryonic germ layers that later originate mature cells of an adult organism. In addition, ESCs are easily propagated and differentiated in vitro, and in suspension, ESCs tend to form embryoid bodies (EBs) and complex structures that mimic mini-organs with oriented axes, resembling developing embryos [3].

Furthermore, in vitro culture of ESCs recapitulates the signaling pathways involved in the neuronal phenotype determination that occurs during central nervous system (CNS) and embryo development [4–6]. During brain development, the temporal and spatial generation of different neuronal types from neural precursor cells (NPCs) occurs through successive stages in which neural stem cells

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(NSCs) differentiate into NPCs and then into neuroblasts. During these developmental processes, environmental and cellular signaling molecules control proliferation, survival and cell fate choice. Many different subtypes of neurons, such as serotoninergic, dopaminergic, GABAergic, cholinergic and glutamatergic neurons, are derived from NPCs. The mechanisms, by which stem cells produce a variety of neuronal subtypes, are still under investigation [7].

Even at the earliest stages of brain development, specific neurotransmitter receptors are expressed by the progenitor cells of the developing CNS. Many studies have provided evidence that signaling initiated by extracellular nucleotides (EXNs), such as ADP, ATP and UTP, is important for neurogenesis and neuronal differentiation [8–14]. The mechanisms underlying the effects of these molecules have never been thoroughly investigated. However, they will be analyzed in detail in the present work.

EXNs bind to and activate purinergic receptors, which are divided into two different subtypes according to their ligands: P1 and P2 receptors [15, 16]. P1 receptors are metabotropic receptors activated by adenosine. P2 receptors are subdivided into two classes based on their structural properties: (1) P2X receptors are ionotropic ligand-gated receptors formed by three subunits of the P2X1–P2X7 subtypes that are permeable to cations ($Na^+/K^+/Ca^{2+}$) and are activated by ATP and its analogs, and (2) P2Y receptors are metabotropic receptors (of which the P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 subtypes are Gq/G11 protein-coupled, and P2Y12-14 receptors are coupled to Gs or Gi proteins) that trigger Ca^{2+} release from the endoplasmic reticulum (ER) through inositol 1,4,5-triphosphate (IP3) [15, 17, 18]. Both P2X and P2Y receptor activation induces oscillations in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), promoting specific signal transduction cues and cellular responses.

Neurons undergo Ca^{2+} influx and mobilization of intracellular Ca^{2+} stores, resulting in two main types of spontaneous $[Ca^{2+}]_i$ patterns depending on neural gene expression and activity. As described by Spitzer et al. [19] in studies on *Xenopus laevis*, wave-like oscillations show small $[Ca^{2+}]_i$ amplitudes, low frequencies and an approximate duration of 30 s. These oscillations occur upon activation of ryanodine receptors (RYRs) and IP3 receptors (IP3Rs) under resting potential conditions and spread to neighboring cells by gap junctions, thereby losing strength as they depart from the origin point [19, 20]. Spike-like, high-amplitude oscillations depend on extracellular Ca^{2+} influx through activation of voltage-gated Ca^{2+} channels (VGCCs), including L-type channels; the oscillations last ~10 s and subsequently propagate throughout the entire length of the excitable cell [21]. Neurons in culture exhibit low-frequency spike transients, as do stem cell models subjected to neuronal differentiation [9, 22], in order to regulate gene expression and to progress neural

differentiation and phenotypic specification through transcription factor recruitment [23–26].

The importance of VGCCs in early development has already been reviewed [27]. Calcium channels are expressed from the oogenesis stage on, earlier than egg fertilization. In addition, they play important roles in neurotransmitter release and are located at synaptic terminals [28]. Cav2.1 is a N-type Ca^{2+} channel and is more highly expressed in the early stages of development. Moreover, activation of the N-type calcium channel and P/Q-type calcium channel (Cav2.2) subunit interaction with laminin 2 causes an influx of calcium through the growth cone, inhibiting synapse formation [29]. R-type Cav2.3 channels may control neuronal viability, as they are implicated to participate in neurodegenerative diseases [30–32]. Expression downregulation of L- and N-type calcium channels in favor of P/Q- and R-type calcium channels during neuronal maturation has already been shown [33].

Direct modulation of transcription factors by spontaneous $[Ca^{2+}]_i$ oscillations, providing a cellular clock, has never been studied in real time due to methodological limitations. Therefore, we developed a time-lapse imaging approach that records $[Ca^{2+}]_i$ and transcription factor (neurogenin (Ngn) 2 and ASCL1) expression in real time using transgenic cell lines specifically designed for this procedure. The obtained results show that modulation of $[Ca^{2+}]_i$ oscillations and ASCL1 expression patterns increases the yield of GABAergic neurons.

Huntington's disease (HD) is characterized by substantial loss of GABAergic neurons in the basal ganglia leading to motor, mood and cognitive impairments as well as to disorganized and uncontrolled movements [34, 35]. HD is an autosomal dominant inherited disease caused by at least 35 repetitions of the N-terminal CAG trinucleotide (coding for glutamine) in the huntingtin gene (Htt) [36]. Recent studies have correlated longer CAG repeats to earlier symptom onset and a possible neurodevelopmental origin [37].

Here, VGCC- and purinergic receptor-induced modulation of $[Ca^{2+}]_i$ oscillations in neurogenesis and HD was studied in mouse CRISPR/Cas9-modified ESCs and induced pluripotent stem cells (iPSCs) from HD patients induced to undergo neuronal differentiation. Our findings highlight the importance of EXNs and $[Ca^{2+}]_i$ oscillations in cell fate commitment to GABAergic neurons, providing a pharmacological target for HD therapy.

Materials/subjects and methods

Culture and differentiation of E14Tg2A mouse ESCs

The commercially available E14Tg2A ESC line (ATCC® CRL-1821™) was first isolated by Hooper et al. [38] and

further characterized by Magin et al. [39]. The cells were grown as described by Fornazari et al. [40]. For induction of EB formation (day 0), 5×10^6 cells were cultured for 48 h in 90×15 mm^2 nonadherent plates in DMEM supplemented with 20% FBS, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, 2 mM sodium pyruvate, 1% nonessential amino acids and 10 mM HEPES (pH 7.4), at 37 °C in a water-saturated atmosphere containing 5% CO$_2$. Following substitution of the culture medium (day 2), the cells were cultured as a suspension for 4 additional days (until day 6) in the presence of 5 μM *all-trans* retinoic acid (RA) to induce neuronal differentiation. The EBs were seeded in 125 mm^2 adherent cell culture flasks previously coated with 50 μg/ml laminin, and grown for an additional 2–12 days (days 10–20) in DMEM/F12 (Gibco) supplemented with FGF-β (10 ng/ml, Sigma-Aldrich) and N2 supplement (1% v/v, Gibco Thermo Fisher Scientific).

Protein extraction and western blotting assays

For preparation of cell lysates, undifferentiated and neuronal-differentiated E14Tg2A cell lysates were prepared in the presence of a protease inhibitor cocktail (Thermo Fisher Scientific) and phosphatase inhibitors (2 mM ortho-vanadate and 5 mM sodium fluoride, Thermo Fisher Scientific) as described previously [41, 42]. Thirty micrograms of protein in sample buffer was separated by SDS-PAGE and transferred onto nitrocellulose membranes (Thermo Fisher Scientific) as described previously [42]. The membranes were then incubated with primary antibodies against phospho-S133 CREB (monoclonal rabbit, 1:500, Abcam), hnRNP K (monoclonal mouse, 1:1,000, Santa Cruz Biotech) and β-actin (1:1,000, Sigma-Aldrich) overnight at 4 °C. The membranes were then washed and probed with the appropriate secondary antibodies (Alexa Fluor 488 or 647; Life Technologies) for 1 h under agitation at room temperature. The primary and secondary antibodies were diluted in 1% BSA and TBS-T. The membranes were washed in TBS-T and scanned with a Typhoon Imager (GE Healthcare). The resulting bands were subjected to densitometric analysis using the ImageJ software. The phosphorylated CREB staining intensities were normalized to the β-actin levels, and then the nuclear phosphorylated CREB levels were normalized to the total phosphorylated CREB levels (cytosolic plus nuclear).

Ca^{2+} imaging in single E14Tg2A ESCs

Undifferentiated and differentiated E14Tg2A ESCs were loaded with 5 μM Fluo-3AM, and Ca^{2+} imaging was performed as previously described [43]. Ca^{2+} influx was monitored in cells stimulated with 10 μM BzATP (a P2X7 receptor agonist), 1 μM 2SUTP (a P2Y2 receptor agonist),

1 μM thapsigargin (an ER Ca^{2+} store depletor), 2 mM EGTA (an extracellular Ca^{2+} chelator), 1 μM israditine (a L-type VGCC blocker) or 500 nM xestospongin C (XeC; an IP3 receptor inhibitor). At the end of the experiment, ionomycin was used to determine maximal fluorescence and cell viability followed by 50 μM EGTA to determine minimal fluorescence values after ionomycin application. In total, 40 cells were analyzed for each data point, and changes in [Ca^{2+}]$_i$ were determined as mean variations between the Fluo-3 fluorescence intensities obtained during the stimulus (F) and the resting state (Fo), normalized by the basal fluorescence (Fb), as follows: (F-Fo)/Fb. For quantification of [Ca^{2+}]$_i$ levels, we used the following formula: [Ca^{2+}]$_i$ = K$_d$ × (Fb-Fmin)(Fmax-Fb), where Fmin is the fluorescence intensity of the indicator in the absence of Ca^{2+} (EGTA), Fmax is the fluorescence of the Ca^{2+}-saturated indicator (ionomycin), Fb is the emitted fluorescence intensity under basal conditions, and K$_d$ is the dissociation constant for Fluo-3AM-Ca^{2+} binding, which is 325 nM. Mean values ± standard errors of the mean (SEM) values were plotted and analyzed by one-way ANOVA with a significance threshold of $p \leq 0.05$.

Ca^{2+} measurements by microfluorimetry

Changes in [Ca^{2+}]$_i$ were determined by microfluorimetry using the FlexStation III (Molecular Devices Corp.) following the instructions of the manufacturer [44]. Briefly, for undifferentiated cells, the cells were seeded the night before starting the experiment at a density of $3–5 \times 10^4$ cells/well. For 8-day differentiated cells, 5 EBs/well were seeded in 96-well black microplates with clear bottoms, with 100 μl of cell culture medium per well. The cells were incubated with reagent from a FLIPR Flex Station Calcium Assay Kit (Molecular Devices Corp.) as previously described [42]. Modulators (BzATP, ATP, KCl and BayK8644) were applied to the cells, and [Ca^{2+}]$_i$ transients were determined as the peak fluorescence intensity minus the basal fluorescence intensity using the SoftMax2Pro software (Molecular Devices Corp). Data are expressed as the mean values ± SEM.

Analysis of membrane potential variation by microfluorimetry

Changes in membrane potential were determined by microfluorimetry using the FlexStation III microplate reader and the FLIPR Membrane Potential Assay Kit (Molecular Devices Corp) according to the instructions of the manufacturer, as described previously [45, 46]. Results obtained with this kit are in good correlation with those obtained in patch-clamping assays. Samples were measured at 1-s intervals for 120 s after 30 s of monitoring basal fluorescence intensity as a measure of the membrane potential of resting cells. A depolarizing agent (KCl and either BzATP

or ATP) was added to the cells in the presence or absence of the L-type VGCC inhibitor isradipine (1 µM). Responses to agent addition were calculated as the peak fluorescence minus the basal fluorescence. Fluorescence intensity was determined using the SoftMax2Pro software (Molecular Devices Corp.). Data are expressed as the mean values ± SEM.

Immunofluorescence staining assay

Immunofluorescence detection of proteins was specifically performed by immunolabelling various differentiation stages, and E14Tg2A cells were grown and induced to differentiate on rounded coverslips (1 cm in diameter). Cells were prepared as described previously [42] and exposed to primary antibodies, including rabbit polyclonal anti-TUJ1 (1:200, Millipore), mouse monoclonal anti-Doublecortin (DCX) (1:100, Santa Cruz Biotech), rabbit polyclonal anti-GAD65/67 (1:500, Millipore), GFAP (1:1,000, Dako), anti-tyrosine hydroxylase (TH; 1:100, Santa Cruz Biotech.), anti-GABA (1:100, Santa Cruz Biotech.), MASH1/ASCL-1 (1:100, Santa Cruz Biotech.), anti-phosphorylated CREB (1:250 Abcam), anti-L-type VGCC (1:500 Abcam) and anti-Nestin (1:1,000 Millipore) antibodies. As secondary antibodies, we used Alexa Fluor 488- or 555-conjugated goat anti-mouse or anti-rabbit antibodies (1:800, Sigma-Aldrich). In control experiments, the primary antibodies were omitted, and immunostaining was never detected. Counterstaining of cell nuclei was achieved with 6-diamidino-2-phenylindole (DAPI, 300 nM). Samples were examined on a confocal microscope (Zeiss LSM 780-NLO Multiphoton) and analyzed with LSMib software (Zeiss) and StrataQuest (TissueGnostics, Austria) software.

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from undifferentiated cells and ESCs subjected to 8 days of neuronal differentiation using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. All samples were processed, and real-time PCR was performed as previously described [42] with primers in Table 1. The comparative $2^{-\Delta\Delta CT}$ method was employed for relative quantification of gene expression as described previously [42, 45] using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression as an internal standard for normalization.

pNgn2-luciferase (LUC) transgenic ESCs

To track the real-time expression of the basic helix-loop-helix (bHLH) transcription factor Ngn2 during neurogenesis, we derived ESCs expressing LUC2 as a reporter gene fused to the promoter sequence of Ngn2 [47]. The construct containing the promoter and exons 1 and 2 of Ngn2 fused to luciferase (pNgn2-LUC2) as a BAC DNA was described first by the Kageyama group; we subcloned this construct into a Tol2-pPGK-mCherry-NLS-IRES-Puro backbone vector (T2a-MC-Puro vector). Subcloning was performed in two steps. The first involved 5′ and 3′ pNgn2-LUC2 homology arm (HA) synthesis by PCR using the primers 5′HA forward (gtcgacC-TAATTAGACAGATCTGAGTGCGG), 5′HA reverse (gcg gccgcTGCTAACCATGAAGACCAGAGG), 3′HA forward (gcggccgcCTGGGAACACAGGGCTATGC), and 3′HA reverse (gaattcCCATCTAGAAGAACAGAAGAACGGG) and insertion into the T2a-MC-Puro vector by SalI and BamHI enzymatic digestion followed by ligation into the multiple cloning site using a DNA Ligation Kit, Mighty Mix (TaKaRa). The second cloning step involved substitution of the Ngn2-LUC2 sequence into the T2a-MC-Puro vector through homologous recombination with the homology arms.

DNA sequences were identified with the BigDye Terminator v3.1 Cycle Sequencing Kit on the ABI PRISM 3100 Genetic Analyzer using the following primer sequences: 1-luc2_5F: GTGGACATTACCTACGCCGAG, 2-luc2_5R: CTTCATAGCTTCTGCCAGCCG, 3-Seq7_luc2: TGCTCA TGTACCGCTTCGAGG, 4-luc2_3F: TGTGTTCGTGGAC GAGGTGCC, 5-luc2_3R: GTCCAACTTGCCGGTCAGT CC, 6-Ngn2_rev: ATCAAGAAGACCCGCAGGCTC, and 7-Ngn2_for: CTCTAGATACAGTCCCTGGCG. The sequences were checked for mutations by alignment analysis using a plasmid editor (ApE; RRID: SCR_014266).

The plasmid Ngn2-LUC-t2a-mCherry-Puro with a size of 15 kb was transfected into undifferentiated ESCs using the ViaFect Transfection Reagent (Promega) and 3.6 µg of DNA template in Opti-MEM (Gibco). At 4 days post-transfection, puromycin (1 µg/ml) was added for 7 days to the cell culture to select cells stably expressing pNng2-LUC2. Following selection, $\sim 4 \times 10^5$ cells were double-sorted for positive mCherry expression with a FACSCalibur (BD Biosciences) instrument using the 561 nm yellow/green laser excitation and a 610/20 bandpass emission filter.

ASCL-1-LUC NPCs from mouse telencephalons

For ASCL-1 expression analysis, NPCs were used, which had been derived from transgenic mouse embryo telencephalons by nuclear microinjection of one bacterial chromosome (BAC DNA) containing an insert composed of the ASCL-1 promoter region and ASCL-1 exons fused to LUC2, as previously established by Imayoshi et al. [47].

Indirect ASCL-1 and Ngn2 expression detection by time-lapse luminescence recording

After incubation for 1 h with the Ca^{2+} indicator Fluo-3AM, transgenic cells expressing Ngn2 or ASCL-1 fused to

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Table 1 Sequences of the primers used for real-time PCR.

Gene	Forward (5′-3)′	Reverse (5′-3′)	bp
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC	177
SSEA-1	CGGACCGACTCGGATGTCT	TTGGATCGCTCCTGGAATAGA	58
Oct-4	ATG CCG TGA AGT TGG AGA AG	TGT ACC CCA AGG TGA TCC TC	123
S100β	GAGCAGGAAGTGGTGGACAA	CACTCCCCATCCCCATCTT	59
TH	CCTTCCGTGTGTTTCAGTGC	TCAGCCAACATGGGTACGTG	112
ChAT	CCCAACCAAGCCAAGCAATC	GGATAGGGGAGCAGCAACAA	113
GAD65	CACCGAGCTGATGGCATCTT	GGGCGCAGGTTGGTAGTATT	84
NMDAR	TGGGAAGTGTACGGTGCTTC	CGATCTGACGCTCCAAATGC	86
V-Glut2	GACCCTGAGGAAACAAGCGA	TCCTGTGAGGTAGCACCGTA	131
5-HT	CAAAGGGGACCTTCCTCTGC	CATCTTGCGCTTTGCCTCAG	103
Dcx	GAGTGGGGCTTTCGAGTGAT	AAAGAAAGCCGTGTGCCTTG	78
MAP-2	TCCTCCAAAGTCCCAGCTA	CCGGCAGTGGTTGGTTAATA	245
TUJ1	AGA CCT ACT GCA TCG ACA ATG AAG	GCT CAT GGT AGC AGA CAC AAG G	110
Nestin	GAG AGT CGC TTA GAG GTG CA	CCA CTT CCA GAC TCC GGG AC	241
Ngn2	TCGGCTTTAACTGGAGTGCC	GTGTGTTGTCGTTCTCGTGC	94
GFAP	AAGAGTGGTATCGGTCCAAGTTTG	CAGTTGGCGGCGATAGTCAT	107
P2X2	TCCCTCCCCCACCTAGTCAC	CACCACCTGCTCAGTCAGAGC	149
P2X3	CTGCCTAACCTCACCGACAAG	AATACCCAGAACGCCACCC	150
P2X4	CCCTTTGCCTGCCCAGATAT	CCGTACGCCTTGGTGAGTGT	149
P2X5	GGATGCCAATGTTGAGGTTGA	TCCTGACGAACCCTCTCCAGT	81
P2X6	CCCAGAGCATCCTTCTGTTCC	GGCACCAGCTCCAGATCTCA	152
P2X7	GCACGAATTATGGCACCGTC	CCCCACCCTCTGTGACATTCT	171
P2Y1	GAGACACGAGTTTCTGAAGGC	CAGGGATGTCTTGTGACCATGT	70
P2Y2	CTGATCAGGTCCAGGGCAAT	GTATCCCAGTTCGTCCCCCT	87
P2Y4	AAGGGTGGTGGTGGTACTCC	AGGAGCACCATCTTAGTTCCAG	100
P2Y6	TTGCATGAGACAGACTCTCCG	ATGGTGCCATTGTCCTGCTC	70
P2Y12	GTGCAAGGGGTGGCATCTAC	CCAAACTGGAAAAACAGGGGT	70
P2Y13	AACAAAGCTGATGCTCGGGA	CAGCTGTGTCATCCGAGTGT	94
P2Y14	TTCGTCCGGCAGCTGTAGT	ACATTGCCAGAATCCCCTACAC	61
cav1.1	CGTCATCGGCAGCATCATTG	ATCTGGGTCAACGTTCCCG	109
cav1.2	CCTCATCGTCATTGGGAGCA	TCCTCTGCACTCATAGAGGGA	248
cav1.3	CGCAACACGATACTGGGCTA	TAGTTCCTGCAGAAAGCCCC	119
cav1.4	TGGCAACTACATCCTGCTGAAC	GAGGGTTTCCTTCACTGCTCT	116
cav 2.1	GAGGGTTTCCTTCACTGCTCT	TCGATCATCTTGATCACCATCTCA	102
cav 2.2	TGCGTTCTCGAGCTTCATGG	CCGCTTGATGGTCTTGAGGG	103
cav2.3	CCAGCAACAGATTGAGCGAG	CGAAGCACTTCCAAGGCTGA	117
hP2X3	ACGCCAACAGAGTCATGGATG	CGCACTGGCTGTCTGATACA	159
hP2X4	GAGATTCCAGATGCGACCACT	ACCCGTTGAAAGCTACGCAC	112
hP2X6	TCAACTTCTCTAAGTCCAATGCC	CAGTAGGGGCTGAATTGTGGT	88
hP2X7	GTGCCGAAAACTTCACTGTGC	CTGGCAGGATGTTTCTCGTGG	81
hP2Y1	GGGATGCCATGTGTAAACTGC	CGCTGATACAGATCGCATTCTT	159
hP2Y2	CGTGGCCTACAGCTCAGTC	GTGACGTGGAATGGCAGGAA	207
hP2Y4	TGGCATTGTCAGACACCTTGT	AAAGAAAGCGGACGAACTTGC	114
hP2Y6	GTGTCTACCGCGAGAACTTCA	CCAGAGCAAGGTTTAGGGTGTA	159
hP2Y11	AGCTCCTATGTGCCCTACCA	GCGGCCATGTAGAGTAGAGG	197
hP2Y12	TTTGTGTGTCAAGTTACCTCCG	CTGGTGGTCTTCTGGTAGCG	101
hP2Y14	AATCTAGCCGCAACATATTCAGC	GTCTGACTCTTTGTGTAGGGGAT	94

luciferase were incubated in culture medium without growth factors or serum for differentiation induction and 1 mM luciferin (Nacalai Tesque). The cells were recorded in 5% CO_2 at 37 °C under a fluorescence microscope coupled to a CCD camera (iKon-M DU934P-BV). Ca^{2+} measurements were obtained at 1 frame/5 s, while luminescence was recorded at 1 frame/10 min. ImageJ software was used for image analysis; a spike noise filter was applied to stack images to remove cosmic ray signals, and then a Savitzky Golay temporal filter was used to obtain clear dynamic expression results.

In vitro model of HD/human iPSC culture and differentiation

Human NPC lines derived from healthy donor iPSCs (hN8; ax0018) and from HD patients (HD; ax0211, 45CAG repetition) were obtained from the Axol Bioscience Stock Bank. The cells were cultured according to the seller's recommendations. Differentiation of these cells into medium spiny GABAergic neurons was induced following previously described protocols [48].

Murine model

The plasmids pEGFP-Q74 (Plasmid # 40262, Addgene) for overexpression of mutated Htt (mHtt) with 74 repetitions of glutamine (Q74) and pEGFP-Q23 (Plasmid # 40261, Addgene) for overexpression of wild-type (WT) Htt with 23 repetitions of glutamine (Q23) were obtained from Prof. Dr. Soraya Smaili, Federal University of São Paulo, Brazil.

For transfection of the E14Tg2A strain, we incubated the following mixture for 20 min: 3 µg of plasmid, 11 µl of ViaFect™ Transfection Reagent (Promega), and 500 µl of Opti-MEM (Gibco). After incubation, the mixture was transferred onto a 100 mm² plate containing suspended E14Tg2A cells in the absence of leukemia inhibitory factor for the formation of transfected EBs. The medium was changed every 2 days until 8 days of differentiation, when the EBs were seeded in 35 mm plates for morphological analysis of migration and differentiation.

We used a kit for Htt mouse gene knockout via CRISPR/Cas9, KN308042 (OriGene Technologies) for transfection of the E14Tg2A strain. For transfection of cells with Htt gene knockout plasmids we incubated the following reaction for 20 min: 1 µg of plasmid containing Cas9 sequences and guide RNA, 3 µl of ViaFect™ Transfection Reagent (Promega), and 500 µl of Opti-MEM (Gibco). After incubation, the mixture was transferred onto a 35 mm plate containing E14Tg2A cells at 70% confluence. The medium was changed the next day, the cells were subcultured for 10 passages as detailed in the manual, and the protein samples were collected for Western blot analysis for

determination of Htt protein levels. We successfully established a pair of Htt knockout ($Htt^{-/-}$) strains, since Htt expression was not detected in cells transfected with guide RNA 1 (G1) or with guide RNA 2 (G2), whereas it was detected in untransfected cells or cells transfected with the scrambled control (SCR).

Statistical analysis

Comparisons between experimental data were performed with one- or two-way analysis of variance followed by the Bonferroni posttest using the GraphPad Prism 5.0 software (GraphPad Software, Inc.). The criteria for statistical significance were set at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). The samples followed normal distribution. The number of independent experiments is described in the figure legends.

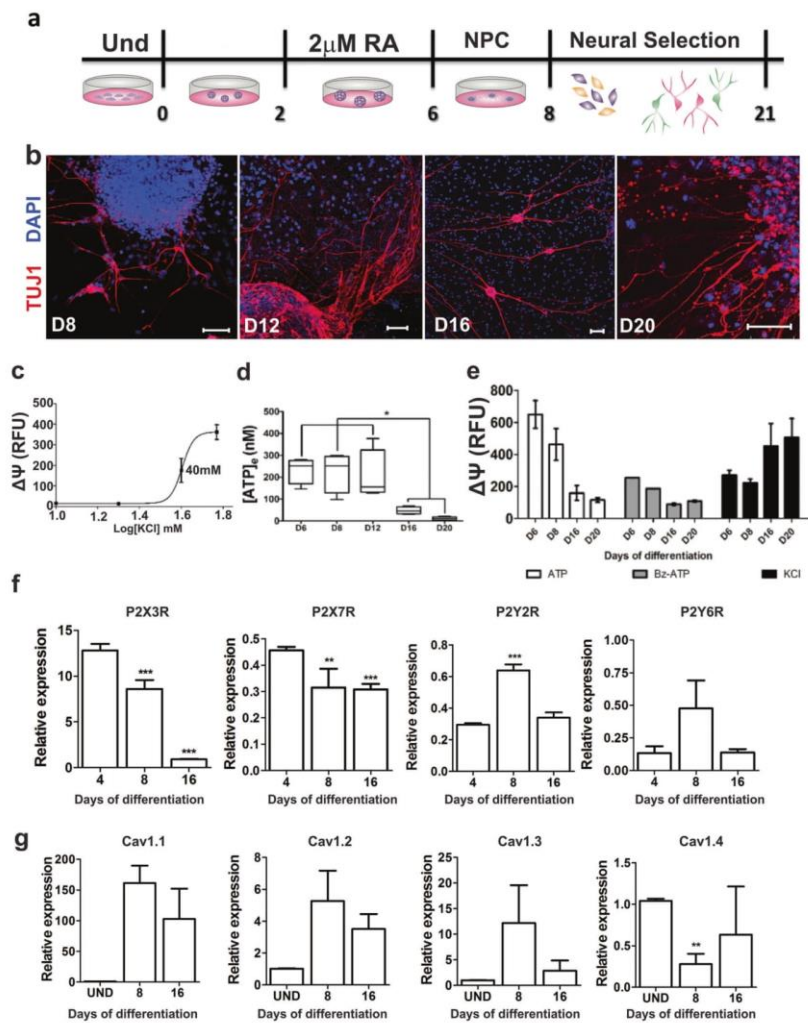## Results and discussion

Pan-neural differentiation of ESCs

We used a differentiation protocol based on EB formation from mouse ESCs that includes induction of neuroectodermal differentiation by *all-trans* RA followed by selection and differentiation of NPCs (Fig. 1a). ESCs are easily cultivated in vitro and tend to form EBs that resemble in vivo embryos due to the high cellular complexity and organized hierarchy within the tissue [3]. In addition, EBs also recapitulate signaling pathways involved in CNS and embryo development [4, 5].

The highest expression of NPC-specific nestin began on day 8 and lasted until day 12, and DCX was also expressed (Supplementary Fig. 1a–c). Gene expression of the neuron markers TUJ1 and MAP-2 started on day 8 and increased during differentiation (Fig. 1b and Supplementary Fig. 1c). GFAP expression analysis showed that gliogenesis had a later onset approximately on day 16 (Supplementary Fig. 1c, d).

Various neuronal phenotypes were observed, such as the cholinergic, glutamatergic, serotoninergic, GABAergic and dopaminergic types. Membrane depolarization occurred upon KCl administration, with the half-maximal response triggered by 40 mM KCl (Supplementary Fig. 1e–g and Fig. 1c). Altogether, these data allowed us to set a time window to investigate neurogenesis from days 6–8, as previously described [49, 50].

Involvement of EXNs and VGCCs in pan-neural differentiation

Early in development, EXNs are involved in neurogenesis and neuronal differentiation [8–12]. However, studies have

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not investigated the molecular mechanisms underlying neurogenesis control in detail. In this study, we showed that the extracellular ATP concentration was augmented from days 6–12 (Fig. 1d). ATP binding to ionotropic P2X receptors results in increased cationic currents, including Ca^{2+} currents, with subsequent signal transduction, depolarization even at a low ATP concentration (100 nM), and activation of L-type VGCCs [50]. For instance, we observed that ATP and BzATP (an agonist of the P2X7 receptor) depolarized mainly NPCs in the early stages of neuronal differentiation, but not terminally differentiated cells (Fig. 1e). Interestingly, the depolarization induced by KCl mainly affected cells in later stages of differentiation.

Gene expression analysis showed downregulation of P2X3 and P2X7 purinergic receptors (Fig. 1f,

◀ **Fig. 1** Roles of extracellular nucleotide receptors and voltage-gated calcium channels in RA-induced neural differentiation of ESCs. **a** Schematic design of the differentiation protocol. ESCs treated with 2 μM *all-trans* RA and suspension-cultured formed EBs resembling early developing embryos. On day 6, the cells were seeded onto laminin-coated dishes and cultured in the absence of serum to differentiate into NPCs. **b** Expression of the neuronal marker TUJ1 detected by immunofluorescence microscopy during differentiation is shown in red, with nuclei in blue (DAPI) (days 8, 12, 16, and 20). **c** Membrane potential variation in cells after 16 days of differentiation recorded by microfluorimetry following stimulation with KCl. **d** Extracellular ATP concentrations measured by a firefly luciferase-luciferin assay after 5 min of incubation of cells at various stages of differentiation (days 6, 8, 12, 16, and 20). **e** Membrane potential variation in cells after 6, 8, 16 and 20 days of differentiation as determined by microfluorimetry following stimulation with 100 μM ATP, 10 μM BzATP or 60 mM KCl. **f** Gene expression of purinergic receptors (P2X3, P2X7, P2Y2 and P2Y6) on different days of differentiation (days 4, 8, and 16). The data are representative of at least three independent experiments and are shown as mean values ± SEM; one-way ANOVA followed by the Bonferroni post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **g** mRNA expression of L-type VGCCs (Cav1.1-Cav1.4) on different days of differentiation (undifferentiated, days 8 and 16). The data are representative of at least three independent experiments and are shown as mean values ± SEM; one-way ANOVA followed by the Bonferroni post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Supplementary Fig. 2a) and L-type VGCC cav1.4 (Fig. 1g, Supplementary Fig. 2b) during the differentiation process, while transient expression upregulation of P2Y2 and P2Y6 receptors and L-type VGCC cav1.1, cav1.2 and cav1.3 appeared on day 8 of differentiation, which is the NPC stage. N-type and P/Q-type calcium channels were also present in the samples at later stages of the differentiation process (Supplementary Fig. 2), corroborating the involvement of these ion channels in neurotransmitter release and synapse formation [32].

Roles of P2X7 and P2Y2 receptors in cell fate determination

Previously, in our work, we described P2X7 receptor activity and expression inhibition during neuroblast differentiation; however, we did not study P2Y2 receptors in this regard. Therefore, P2X7 and P2Y2 receptors were chronically activated during days 6 to 16 of differentiation. We found that the glutamatergic (V-Glut2 and NMDAR), dopaminergic (TH) and serotoninergic (5-hydroxytryptamine, 5-HT) neuronal marker expression was diminished in the condition of P2Y2 receptor stimulation when compared with the control condition (Fig. 2a). Notwithstanding, augmented expression levels of the GABAergic neuronal marker occurred in cells treated with 2SUTP, a selective agonist of the mouse P2Y2 receptor (Fig. 2a, b). We previously confirmed the concentration of BzATP for selectively activating P2X7 receptors [42].

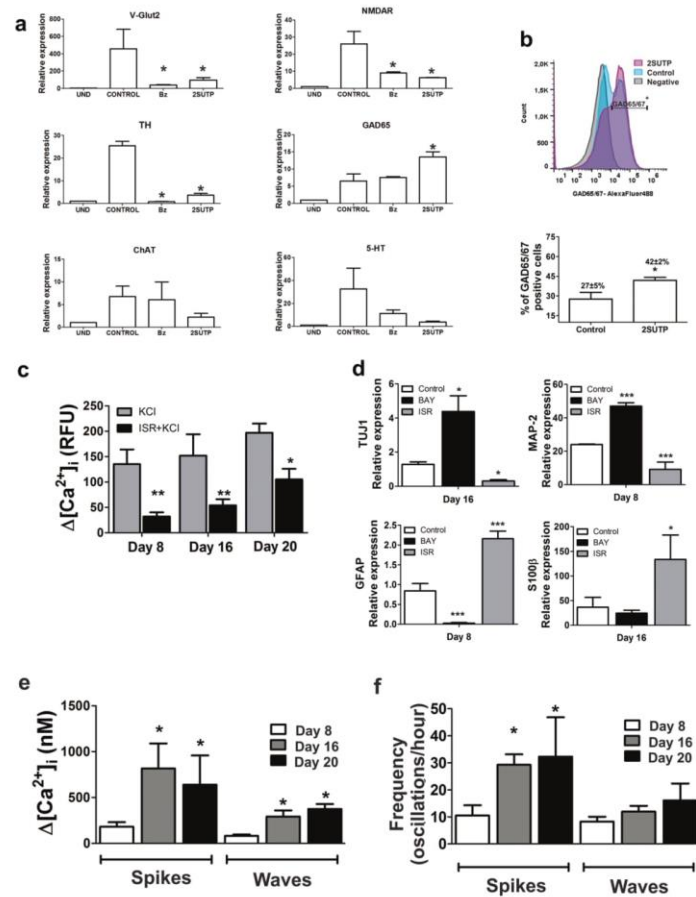## Role of L-type VGCCs in glial versus neuronal differentiation

In addition to exhibiting increased L-type VGCC subtype expression, neuronal-differentiating cells (day 8) were more sensitive at this stage to the L-type channel modulators, both the activator BayK8644 (Supplementary Fig. 2c) or its blocker isradipine plus KCl (Fig. 2c). Furthermore, L-type VGCCs mediated most of the depolarization-triggered Ca²⁺ influx (Fig. 2c). Chronic treatment of cells with BayK8644 beginning on day 6 significantly increased neuronal TUJ1 and MAP-2 expression while decreasing GFAP glial cell marker expression (Fig. 2d). Moreover, isradipine treatment exerted the opposite effect, notably increasing S100β expression; this finding indicated that isradipine directed differentiation into astrocytes (Fig. 2d).

Here, we observed that the ATP levels were higher in the NPC stage than in the neuron stage and that activation of the P2Y2 receptor favored GABAergic neuronal differentiation. Extracellular ATP promoted NPC membrane depolarization, leading to L-type VGCC activation. The resulting Ca²⁺ influx induced neuronal differentiation. Moreover, isradipine treatment notably increased S100β expression, augmenting differentiation of the cells into astrocytes (Fig. 2d). Accordingly, D'Ascenzo et al. [51] showed that L-type VGCC inhibition decreased neuronal differentiation but did not investigate glial differentiation.

We have previously described P2X7 receptor activity during ESC proliferation and inactivation of these receptors during neurogenesis [42]. The P2Y2 receptor is expressed in mammalian embryonic regions involved in the formation of spinal motor nerves and is associated with sensory neuron development [52]. In vitro P2Y2 receptor agonism in mouse NSCs increased migration, indicating the possible involvement of this receptor in NSC migration in the adult brain [50]. Moreover, neuronal-differentiating P19 cells express active P2Y2 receptor [8, 53]. Consequently, we speculate that the P2X7 receptor favors gliogenesis and inhibits neurogenesis, while the P2Y2 receptor promotes neurogenesis and favors GABAergic differentiation, in parallel with L-type VGCCs, which also induce neurogenesis.

Spontaneous Ca²⁺ oscillations during pan-neuronal differentiation

We analyzed patterns of spontaneous [Ca²⁺]ᵢ events induced during *all-trans* RA neural differentiation as well as mechanisms involved in this process and their consequences. [Ca²⁺]ᵢ transients of EBs recorded for 20 min (Fig. 2e, f, Supplementary Fig. 3) revealed two Ca²⁺ oscillation patterns (Supplementary Fig. 3a, b) with different durations and amplitudes [19, 20]. Spike-like oscillation patterns presented

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500 nM amplitudes with a 15-s duration, while wave-like oscillation patterns consisted of 250 nM amplitudes with a 30-s duration. During neural differentiation, spike-like oscillations increased in both amplitude (Fig. 2e) (from 300 ± 21 nM on day 8 to 800 ± 253 nM on day 16) and frequency (Fig. 2f) (from 10 spikes/hour on day 8 to 32 spikes/hour on day 20). Amplitudes augmented from 250 ± 23 nM on day 8 to 400 ± 33 nM on day 20 (Fig. 2e), and wave-like oscillations (Fig. 2f) increased from 8 spikes/hour on day 8 to 17 spikes/hour on day 20. In NPCs on day 8, the amplitudes and frequencies of the spike- and wave-like oscillations decreased along with cell commitment, differentiation and maturation, and the frequencies and amplitudes of the $[Ca^{2+}]_i$ oscillations were

increased, suggesting a role of these oscillations in the maintenance of neural development.

To further elucidate the mechanisms, by which these oscillations are triggered, XeC or EGTA were applied to seeded EBs on day 8 to reduce intracellular or extracellular $[Ca^{2+}]$, respectively. Treatment with 2 mM EGTA chelated extracellular Ca^{2+} and abolished spike-like oscillations (Supplementary Fig. 3a). XeC blocked intracellular Ca^{2+} release and caused extinction of wave-like oscillations and increased spike-like frequency (Supplementary Fig. 3b-d). On the other hand, treatment with XeC did not block spike-like oscillations and increased spike frequency (Supplementary Fig. 3).

◀ **Fig. 2 Effects of VGCCs and P2Y2 and P2X7 receptor-induced signaling on NPC differentiation. a** Gene expression of neuronal phenotype markers (V-Glut2 and NMDA: glutamatergic; TH: dopaminergic and noradrenergic; GAD65: GABAergic; choline acetyltransferase (ChAT): cholinergic; 5-hydroxytryptamine: serotoninergic) after 16 days of differentiation upon stimulation of P2X7 receptors (10 μM BzATP) or P2Y2 receptors (1 μM 2SUTP). The data are representative of at least three independent experiments and are shown as the mean values ± SEM; one-way ANOVA followed by the Bonferroni post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **b** Flow cytometry analysis results shown as the mean values ± SEM of the percentage of GABAergic neurons (GAD65/67+) after 16 days of differentiation in the presence of a P2Y2 receptor agonist (2SUTP). The values are expressed as the mean values ± SEM of GAD65/67+ cells. **c**. Variations in [Ca^{2+}]$_i$ after stimulation with 60 mM KCl with or without pretreatment with isradipine (an L-type VGCC blocker), as determined by microfluorimetry. The data are representative of at least three independent experiments and are shown as the mean values ± SEM. Relative fluorescence units (RFU). **d** Gene expression of neuronal differentiation markers (TUJ1: neuron; MAP-2: mature neuron; GFAP: glial cell; S100β: astrocyte) after 8 or 16 days of differentiation upon stimulation or inhibition of L-type VGCCs (0.1 μM BayK8644 (Bay) and 1 μM isradipine (ISR)). The data are representative of at least three independent experiments and are shown as mean values ± SEM; one-way ANOVA followed by the Bonferroni post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). GAPDH was used to normalize the real-time PCR data. **e** and **f** Spontaneous [Ca^{2+}]$_i$ oscillations on day 8 of differentiation. Following 20 min of calcium imaging, cells were stimulated with ionomycin (5 μM) and EGTA (50 μM). Spikes (15-s duration) and waves (30- to 60-s duration) were detected. The data are representative of at least 20 cells/ three independent experiment. **e** Analysis of the [Ca^{2+}]$_i$ amplitudes of spike- and wave-like spontaneous [Ca^{2+}]$_i$ oscillations on days 8, 16 and 20 of differentiation. **f**. Analysis of the frequencies of spike- and wave-like spontaneous [Ca^{2+}]$_i$ oscillations on days 8, 16, and 20 of differentiation. The data are representative of at least three independent experiments (20 cells each) and are shown as mean values ± SEM; one-way ANOVA followed by the Bonferroni post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

P2 receptor stimulation triggers oscillations in [Ca^{2+}]$_i$, modulating neural gene expression and activity. As first described in *X. laevis*, wave-like oscillations with small [Ca^{2+}]$_i$ amplitudes, low frequencies and approximate durations of 30 s occur upon activation of RYRs and IP3Rs, while spike-like oscillations last approximately 10 s and subsequently propagate throughout the entire extension of an excitable cell [19, 20]. Accordingly, we observed that both the amplitude and frequency of spike-like oscillations increased during differentiation, while only the amplitude of wave-like oscillations increased (Fig. 2e, f and Supplementary Fig. 3). In later stages of *X. laevis* nervous system development, Spitzer et al. [21] showed that spike frequency decreases with maturation, resulting in abolishment of the glutamatergic phenotype [21–25]. Moreover, wave-like oscillations remained constant during development, although we observed a slight increase on day 20 corresponding to neuronal maturation. Wave-like oscillations may regulate neurite extension during cone growth through activation of calcineurin, as reported for *X. laevis* [21–25].

Intracellular Ca^{2+} release from ER stores triggers wave-like oscillations, while extracellular Ca^{2+} influx triggers spike-like oscillations, suggesting a possible mechanism of regulation between these two oscillation types (Supplementary Fig. 3). In summary, Ca^{2+} inflow results in spike-like [Ca^{2+}]$_i$ oscillations through L-type VGCC activity, and interactions with the EXN system favor neurogenesis. Malmersjo et al. [54] described the presence of rhythmic [Ca^{2+}]$_i$ oscillations induced by VGCC activation in NPCs. Ca^{2+} signaling also modulates gene expression, thus controlling cell fate [19, 55].

P2X7/P2Y2 receptor and L-type VGCC activation induces alterations in spontaneous [Ca^{2+}]$_i$ oscillations that control proneuronal transcription factor expression

Alterations in [Ca^{2+}]$_i$ influence the activity of diverse transcription factors, such as NFAT, DREAM, and CREB, including proneuronal transcription factors of the fundamental bHLH class [56, 57]. The bHLH factors NGN 1, NGN 2 and ASCL-1 are transiently expressed at low levels during neurogenesis, as previously reported [47] and play pivotal roles in neural fate acquisition and neuronal lineage determination and concomitant inactivation of Hes and Id (transcription factors inhibiting differentiation) [58]. This transient expression induces a second wave of expression of bHLH genes, such as the NeuroD/Nex family genes, for neuronal phenotype specification [58, 59].

Therefore, we investigated whether L-type VGCCs, purinergic receptors and Ca^{2+} signaling control transcription of the proneural bHLH family genes Ngn2 and ASCL-1 (Fig. 3). For this purpose, we used a novel approach, in which transgenic NPCs derived from ESCs or from an embryonic brain expressing transcription factors fused to LUC2, were imaged for luminescence together with calcium imaging. This real-time method enables observation of internal structures and cellular processes over time, highlighting dynamic changes with more reliability and greater relevance than fixed-cell microscopy does [60]. Fluo-3 Ca^{2+}-sensitive fluorescence emission was recorded to detect the influence of L-type VGCC and P2X7 and P2Y2 receptor activities on spontaneous [Ca^{2+}]$_i$ oscillations (5 s per frame for 20 min) and on Ngn2 and ASCL-1 expression (10 min per frame for 48 h) in real time. We decided to focus on spike-like activity, once this type was augmented during differentiation (Figs. 2f, 3g).

L-type VGCC inhibition blocked the spike-like oscillations, while P2X7 and P2Y2 receptor activation increased both the frequency and amplitude of these oscillations in NPCs derived from embryonic brains and from ESCs (Fig. 3a, d). Interestingly, P2Y2 receptor activation by 2SUTP triggered [Ca^{2+}]$_i$ oscillations
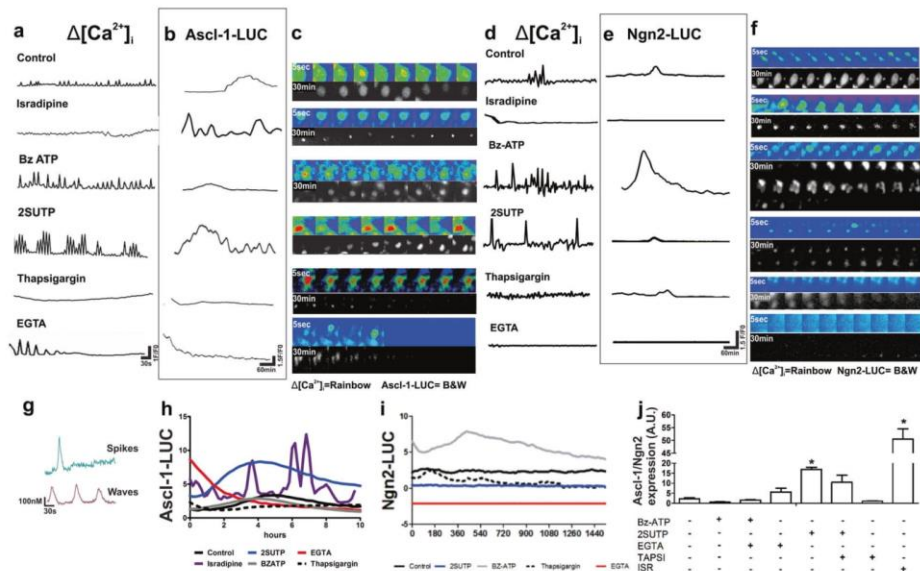
Fig. 3 Effects of P2X7 and P2Y2 receptors on spontaneous calcium oscillations and proneural ASCL-1 and Ngn2 bHLH transcription factor expression during neurogenesis, as determined by time-lapse recording. a Single-cell analysis of spontaneous $[Ca^{2+}]_i$ oscillations by Fluo-3 fluorescence recording of NPCs in the absence of growth factors. The cells were stimulated with BzATP (a P2X7 receptor agonist, 10 µM), 2SUTP (a P2Y2 receptor agonist, 1 µM), thapsigargin (a SERCA inhibitor, 1 µM) or EGTA (a calcium chelator, 2 mM) prior to 20 min of recording at 1 frame/5 s. The data are representative of at least 40 cells/two independent experiments. **b** Single-cell analysis of ASCL-1-LUC expression in NPCs in the absence of growth factors was performed by time-lapse imaging, since luminescence emission directly correlated to ASCL-1 expression. Cells previously recorded for 20 min for measurement of $[Ca^{2+}]_i$ oscillations were subjected to luminescence recording for 48 h (10 min/frame). The cells were treated as described in **a**. **c**. Representative single-cell time-lapse images are shown. Rainbow-pseudocolored images show Fluo-3 dye fluorescence emission, while black and white (B&W) images show ASCL-1 luminescence emission. The time interval between each frame was 5 s for calcium recording and 30 min for ASCL-1 expression experiments. **d** Single-cell analysis of spontaneous $[Ca^{2+}]_i$ variations by Fluo-3 fluorescence recording of NPCs differentiated from transgenic Ngn2-LUC ESCs on day 7 of differentiation. The cells were first stimulated as described in **a**. and then recorded for 20 min at 1 frame/5 s. The data are representative of at least 40 cells/two independent experiments. **e** Single-cell analysis of Ngn2-LUC expression in NPCs differentiated from transgenic Ngn2-LUC ESCs on day 7 of differentiation and measured by time-lapse imaging to evaluate $[Ca^{2+}]_i$ oscillations (**d**) and Ngn2-LUC expression (**e**), as previously detailed in **a**. **f** Overlays of the Ngn2 traces in LUC-transgenic NPCs obtained by time-lapse imaging. The rainbow pseudocolored images show Fluo-3 dye fluorescence emission, while the black and white images show the luminescence of Ngn2-LUC. The time interval between each frame was 5 s for calcium recording and 30 min for Ngn2 expression experiments. **g** Spontaneous $[Ca^{2+}]_i$ oscillations on day 8 of differentiation. Following 20 min of calcium imaging, ionomycin (5 µM) and EGTA (50 µM) were added. Spikes (15-s duration) and waves (30- to 60-s duration) were detected. The data are representative of at least 20 cells/5 independent experiment. **h** ASCL-1 expression analysis in the whole ASCL-1-LUC transgenic NPC population by time-lapse imaging. The cells were treated as described in **a**. **i** Expression analysis in the whole Ngn2-LUC transgenic NPC population by time-lapse imaging. The cells were treated as described in **a**. **j** The cells analyzed in **a** and **d** had the luminescence emission levels of each cell normalized to the constitutive mCherry expression in the same plasmid vector, which was fused to a nuclear localization signaling peptide. For each treatment condition, the ratio between ASCL-1 and Ngn2 expression was defined as the neurogenic index. The data are representative of at least two independent experiments (20 cells each) and plotted as mean values ± SEM; one-way ANOVA followed by the Bonferroni post hoc test (*$p < 0.05$) TAPSI (thapsigargin); ISR: Isradipine.

organized in a burst firing pattern. BzATP increased the frequency and amplitude of the spike-like oscillations; however, depletion of the intracellular Ca^{2+} stores with thapsigargin or depletion of extracellular Ca^{2+} with EGTA decreased their frequency and amplitude (Fig. 3 and Supplementary Fig. 4).

Single-cell analysis of ASCL-1 or Ngn2 real-time expression followed by L-type VGCC blockade or P2X7 and P2Y2 receptor activation showed oscillating ASCL-1 expression levels after isradipine treatment, while 2SUTP treatment increased the expression of ASCL-1 (Fig. 3b, c). These results were corroborated by immunostaining

and real-time PCR (Supplementary Fig. 5a–c). Thus, BzATP controlled and increased the amplitude of Ngn2 expression (Fig. 3e, f). The data obtained from whole-population analysis corroborated the finding that P2Y2 receptor activation increased ASCL-1 expression, while P2X7 receptor activation enhanced Ngn2 expression levels (Fig. 3h, i).

The balance between expression levels of the proneuronal genes ASCL-1 and Ngn2 determines cell fate

The maximum luminescence of ASCL-1- and Ngn2-transgenic cells was determined in the absence or presence of respective agonists and converted into an ASCL-1/Ngn2 ratio, called the "neurogenic index" (Fig. 3j). Activation of the P2Y2 receptor and blockade of L-type VGCCs significantly increased the neurogenic index (Fig. 3j). P2Y2 receptor activation favored differentiation into GABAergic neurons, while L-type VGCC activation promoted neurogenesis. Together, these data indicate that activation of the P2Y2 receptor, along with elevated and sustained expression of ASCL-1 over Ngn2, is important for cell fate determination.

L-type VGCC stimulation triggers CaMKII activation and CREB phosphorylation in neurons [61]. Thus, we investigated the expression of L-type VGCCs in cells on day 8 of differentiation and observed that most cells expressed L-type VGCCs (Supplementary Fig. 5d, upper panel) and that pCREB was localized in the nucleus and in the cytosol (Supplementary Fig. 5d, lower panel).

Furthermore, analysis of the cytosolic and nuclear extracts of cells treated for 15 and 30 min with KCl (60 mM), BayK8644 (100 nM) or isradipine (1 μM) showed an upregulation in the nuclear translocation of pCREB after 15 min of BayK8644 stimulation and a downregulation upon isradipine treatment (Supplementary Fig. 5e, f). However, KCl stimulation alone did not induce translocation, indicating that other signaling pathways triggered by depolarization inhibit pCREB translocation.

This newly developed approach was essential in determining that L-type VGCCs control the stable expression of ASCL-1 by supporting spike-like $[Ca^{2+}]_i$ oscillations and consequent CREB phosphorylation and translocation to the nucleus. Thus, P2Y2 receptor activation led to GABAergic neuron differentiation and spike-like $[Ca^{2+}]_i$ by triggering organized firing bursts that induce the stable expression of ASCL-1. In addition, blockade of L-type VGCCs resulting in oscillating ASCL-1 expression patterns and subsequent differentiation into astrocytes. The results of immunostaining for ASCL-1 in neurogenic sites (Supplementary Fig. 5c) strengthen the indication of the ASCL-1/Ngn2 ratio as a neurogenic index.

The obtained results indicate that L-type VGCCs probably participate in pCREB translocation, since depolarization induced by KCl challenge activates voltage-gated channels, including Ca^{2+} channels. Furthermore, L-type VGCCs are expressed at higher levels and are more sensitive during neurogenesis than during the late stages of differentiation, and activation of these channels partially triggers pCREB translocation. These data corroborate the hypothesis that L-type VGCC activation induces neuronal differentiation. L-type VGCC activation triggered local Ca^{2+} signaling and later CaMKII activation in superior cervical ganglion neurons, leading to CREB phosphorylation [62] and translocation to the nucleus [62].

In a previous work, we observed that P2X7 receptor expression and activity must be downregulated for neuroblast differentiation [42]. We speculate that the disorganized increase in the spike-like frequency upon P2X7R activation may inhibit differentiation by causing bHLH factor expression to become uncoordinated. Imayoshi et al. [47] showed that oscillating ASCL-1 expression is important for NPC maintenance and that the switch to stable expression leads to differentiation. Accordingly, Ciccolini et al. [63] reported that P2 receptor activation increases neurite extension and GABAergic differentiation. GABAergic neurons are important for establishing the brain [64, 65], as evidenced by their presence in the subventricular zone, an NPC- and NSC-enriched region, during neocortex development [66, 67]. Previous studies described that the differentiation of NPCs can be induced by P2Y2 receptor activation or P2X7 receptor blockade [13, 14].

During the early stages of cortical neurogenesis, GABA acts on $GABA_A$ receptors and depolarizes cortical progenitors, leading to increased $[Ca^{2+}]_i$ levels mainly through VGCC activation [68]. Unlike in adulthood, GABA induces excitatory stimuli that promote proliferation [69], migration [70], neurite extension [71] and synapse establishment [72] during cortical neurogenesis. Our study is the first to show a correlation between spontaneous Ca^{2+} signaling and bHLH expression, revealing a mechanism, by which NPCs sense the environment.

Spike-like Ca^{2+} oscillation-triggered P2Y2 receptor activation is absent in HD-patient NPCs

A better understanding of the signaling pathways that control the cell fate determination of GABAergic neurons in the healthy developing brain is needed to enable elucidation of the mechanisms underlying diseases related to GABAergic system deficiency, such as HD, Parkinson's disease and epilepsy [73], in which death of GABAergic neurons in the basal ganglia is a characteristic of HD pathology. To increase our understanding of these pathways, we focused on the roles of the P2Y2 receptor and

spontaneous $[Ca^{2+}]_i$ oscillations in human and mouse HD models. Therefore, we investigated $[Ca^{2+}]_i$ oscillations in HD patients and healthy donor iPSC-derived NPCs.

While morphological observation revealed the presence of rosette-shaped structures in HD-patient and wild-type (WT) cells from healthy individuals, expression analysis of NPC-typical nestin in cultures of undifferentiated cells revealed that there were smaller and fewer colonies of HD NPCs compared to WT NPCs, even when the same numbers of cells were seeded in the dishes (Fig. 4a).

As spontaneous oscillations of $[Ca^{2+}]_i$ may be related to cell fate determination into GABAergic neurons, we assessed spontaneous activity by Fluo-3 indicator fluorescence recording and observed that HD NPCs showed fewer oscillations than WT NPCs did. In addition, the oscillations in HD NPCs were mainly of the wave-type (Fig. 4b–e). Spike-like oscillations were essentially absent in HD cells,

because the amplitudes were very short (<0.3; Fig. 4d) and thus could not be considered oscillations. This phenomenon was probably a result of higher $[Ca^{2+}]_i$ levels in the basal/resting state of HD NPCs compared to WT NPCs (Fig. 4f).

Interestingly, $[Ca^{2+}]_i$ responses evoked by ATP upon GABAergic neuron differentiation induction revealed that compared with WT cells, cells from HD patients had an increased sensitivity towards ATP on day 0 (NPC stage), while no response was detected on day 10 of neuronal differentiation) or on day 20 (in mature neurons) (Fig. 4f). In cells from healthy patients (WT cells), $[Ca^{2+}]_i$ responses decreased with neuronal differentiation (Fig. 4f, g). More-over, cells overexpressing the N-terminal domain of huntingtin, containing a 23 (healthy) or 74 (HD) glutamine residue stretch, were responsive to BzATP and 2SUTP challenge. Notably, cells expressing mHtt showed a
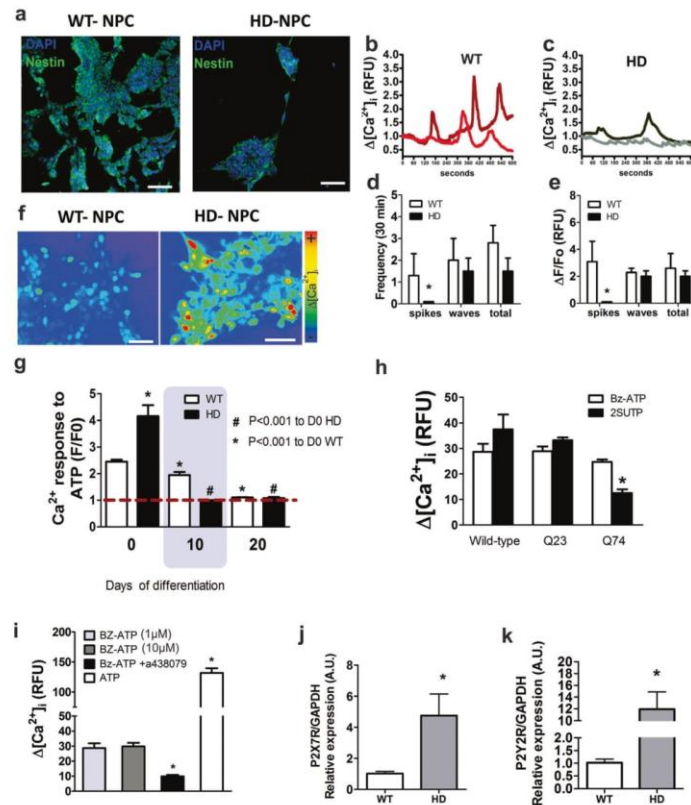
◄ Fig. 4 human NPCs from HD-patient iPSCs do not show spike-like [Ca²⁺]ᵢ oscillations. **a** Expression of the neural precursor marker nestin, as evaluated by immunofluorescence microscopy, is shown in green, while nuclei are shown in blue (DAPI) in HD and WT (hN8) NPCs from patients. **b** and **c** Spontaneous [Ca²⁺]ᵢ oscillations in healthy and HD Fluo-3-loaded NPCs. Following 20 min of calcium imaging, cells were challenged with ionomycin (5 μM) and EGTA (50 μM). Spikes (15-s duration) and waves (30- to 60-s duration) were detected in healthy individuals (WT) and affected patients (HD). Spikes are plotted in dark red and gray, and waves are plotted in light red and black. **d** Analysis of the frequencies of spike- and wave-like spontaneous [Ca²⁺]ᵢ oscillations in NPCs from healthy individuals (WT) and affected patients (HD). **e** Analysis of the [Ca²⁺]ᵢ amplitudes of spike and wave-like spontaneous [Ca²⁺]ᵢ oscillations in NPCs from healthy individuals (WT) and affected patients (HD). **f** HD and WT NPCs loaded with Fluo-3AM were recorded for 3 min using an epifluorescence microscope. **g** Upon induction to GABAergic neuronal differentiation, HD and WT cells loaded with Fluo-3AM were challenged with 100 μM ATP and recorded for 3 min. The data are representative of at least three independent experiments (20 cells each) and are shown as the mean values ± SEM; one-way ANOVA followed by the Bonferroni post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **h** [Ca²⁺]ᵢ response of hNPCs after challenge with a P2X7 receptor agonist (BzATP) and a P2Y2 receptor agonist (2SUTP) by microfluorimetry. Cells were recorded 3 days after transfection with a plasmid for overexpression of WT huntingtin with 23 CAG repetitions (Q23) or mutant huntingtin with 74 CAG repetitions (Q74). WT cells correspond to untransfected cells. **i** [Ca²⁺]ᵢ response of human NPCs derived from iPSCs after challenge with the P2X7 receptor agonist (BzATP) at two different doses (1 and 10 μM), 10 μM BzATP in the presence of the selective P2X7 receptor inhibitor A438079 (1 μM), and ATP (100 μM). **h** and **i** The data are representative of at least three independent experiments. They were analyzed by one-way ANOVA followed by the Bonferroni post hoc test (*$p < 0.05$) and are shown as mean values ± SEM. **j** and **k** Gene expression of purinergic receptors (P2X7 and P2Y2) of hNPCs derived from iPSCs of healthy (WT) and affected subjects (HD). Data were normalized to GAPDH gene expression levels and reported as mean values ± SEM; one-way ANOVA followed by the Bonferroni post hoc test (*$p < 0.05$).

significant decrease in the 2SUTP response, corresponding to impaired P2Y2 receptor activation (Fig. 4h).

ATP activates every P2 receptor. To clarify the participation of P2X7 and P2Y2 receptor activation in hNPCs, BzATP selectivity at 1 μM and 10 μM concentrations was studied in the absence and presence of preincubation with 1 μM of the selective P2X7 receptor inhibitor A438079 (Fig. 4i). As expected, BzATP challenge under P2X7 receptor inhibition did not promote any [Ca²⁺]ᵢ transients, as previously shown by us in mouse NPCs [42]. Nevertheless, real-time PCR analysis indicated that the expression levels of both receptors were upregulated in the HD patient cell line (Fig. 4j, k).

Next, we investigated the roles of Htt and P2Y2 receptors in a murine cellular model of HD. To establish this model, we modified the ESC genome using CRISPR/Cas9, generating a huntingtin knockout (Htt⁻/⁻) cell line and a control line with scrambled sequence (SCR). Western blot analysis detected Htt expression in untransfected (WT) cells and cells transfected with SCR, but not in cells

transfected with either G1 or G2 (Supplementary Fig. 6a and b). The transgenic cell lines were induced to differentiate into GABAergic neurons for 6 days and were transfected with plasmids containing the N-terminus of the Htt sequence controlled by a human cytomegalovirus immediate-early enhancer and promoter and green fluorescent protein (GFP) as a reporter gene. One plasmid had Q23, and the other had Q74; these sequences corresponded to WT Htt and mHtt, respectively (Supplementary Fig. 6a and b). Detection of the reporter gene GFP by immunohistochemistry revealed that plasmid transfection was efficient, since GFP was detected in the Q23 and Q74 (mutant) groups of cells, but not in the blank group (untransfected cells) (Supplementary Fig. 6c).

Flow cytometry analysis revealed that even in the absence of an ectodermal differentiation inducer (RA), the number of cells expressing Q74 was increased by ~20% among neuronal cells (TUJ1-positive cells), and Nestin expression decreased by 66% after 9 days of differentiation (Fig. 5a–c). In addition, cells overexpressing WT Htt (Q23) migrated from EBs more than cells overexpressing mHtt (Q74) (Fig. 5d). Morphological analysis of neurons derived from Htt⁻/⁻ cells and cells transfected with the Q23 and Q74 overexpression vectors indicated that the neurons were healthy and exhibited axonal projections, as did the WT-control neurons (Fig. 5d, e).

Clarifying the effect of the Htt mutation on purinergic signaling, we analyzed the extracellular ATP concentrations in transgenic ESC cultures. There were no differences in extracellular ATP concentrations between WT and mutant NPCs or neurons; however, NPCs presented higher extracellular ATP concentrations than neurons, as was previously observed (Fig. 5f).

Next, we analyzed P2 receptor activation through [Ca²⁺]ᵢ measurements in NPCs (Fig. 5g) and mature neurons (Fig. 5h). ATP (100 μM) induced transients in [Ca²⁺]ᵢ (Δ[Ca²⁺]ᵢ) under the tested conditions. However, Htt knockout decreased Δ[Ca²⁺]ᵢ after administration of ATP (1 μM) or 2SUTP (100 μM). In addition, Htt⁻/⁻ cells expressing the Q74 N-terminus presented no 2SUTP-evoked Δ[Ca²⁺]ᵢ, which indicated that the P2Y2 receptor was not functional in the HD model (Fig. 5g, h), even though we detected P2Y2 receptor expression by immunofluorescence (Fig. 5i) and real-time PCR analysis (Fig. 5j, k). These data corroborate the data obtained with human NPCs, in which P2Y2 receptor activity was impaired, while expression of this receptor was upregulated. This enhanced expression rate may result from a feedback mechanism for compensation of defective activation. HD NPCs also expressed other purinergic receptors, as shown in Supplementary Fig. 7. Compared with WT cells, HD cells showed upregulated expression levels of P2X3, P2X4, P2X6, P2Y1, P2Y4, P2Y11, P2Y12, and P2Y14 receptors, which may reflect defective purinergic signaling.
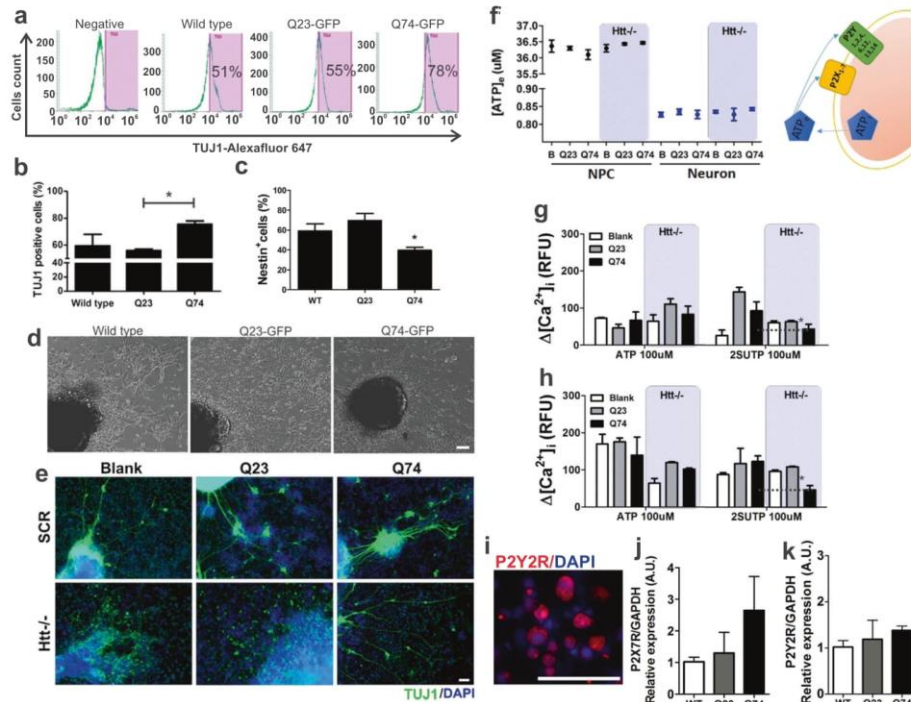
ATP and spontaneous calcium oscillations control neural stem cell fate determination in Huntington's...

Fig. 5 Influence of mHtt overexpression on ESC neuronal differentiation and P2Y2 receptor activity. The ESC genome was modified by CRISPR/Cas9 to generate a huntingtin knockout (Htt-/-) cell line and a control line with SCR. Transgenic cell lines overexpressing WT (Q23-GFP) or mHtt (Q74-GFP) N-terminal Htt sequences were induced to differentiate into GABAergic neurons for 12 days (to the neuron stage). **a**. After 9 days of differentiation, the expression of the neuronal marker TUJ1 (**a** and **b**) and the NPC marker nestin (**c**) was analyzed in cells overexpressing Q23 or Q74 by flow cytometry and immunofluorescence microscopy (**e**). **d** Photomicrography of attached EBs after 9 days of differentiation. Scale bar: 50 μm. f. Extracellular ATP concentrations were measured after 5 min of incubation in cells overexpressing Q23 or Q74 at the NPC and neuron stages of differentiation (days 8 and 12, respectively) by a firefly luciferase-luciferin assay. **g** and **h** $[Ca^{2+}]_i$ oscillations were measured through microfluorimetry in cells overexpressing Q23 or Q74 at the NPC and neuron stages of differentiation (days 8 (**g**) and 12 (**h**), respectively) after challenge with the broad purinergic agonist ATP and the P2Y2 receptor agonist 2SUTP. **i** Immunofluorescence detection of P2Y2 receptor expression (red) in Q74-overexpressing cells after 12 days of differentiation. Nuclei are indicated in blue. Scale bar: 50 μm. Data are representative of at least three independent experiments and are shown as the mean values ± SEMs; one-way ANOVA followed by the Bonferroni post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **j** and **k** Gene expression of purinergic receptors (P2X7 and P2Y2) of mouse NPCs derived from ESCs 3 days after transfection with a plasmid coding for overexpression of WT huntingtin with 23 CAG repetitions (Q23) or mutant huntingtin with 74 CAG repetitions (Q74). WT cells correspond to untransfected cells. Shown data were normalized to GAPDH gene expression and plotted as the mean values ± SEM; one-way ANOVA followed by the Bonferroni post hoc test was used for testing statistical significance (*$p < 0.05$).

Interestingly, in both human and mouse in vitro models, we observed the absence of spike-like $\Delta[Ca^{2+}]_i$ in HD samples and a lack of response to ATP and 2SUTP in neuronal-differentiating cells, corroborating previous findings that the P2Y2 receptor and spike-like $\Delta[Ca^{2+}]_i$ are crucial for the survival of GABAergic neurons. Moreover, P2Y2 receptor expression was detected in every condition on cell membranes, including those overexpressing the N-terminus of mHtt (Q74), indicating posttranslational inactivation of the receptor by Q74 mHtt. Different studies have revealed expression changes in genes related to $[Ca^{2+}]_i$ homeostasis in striatal neurons in the context of HD [74], and our data agree by showing that $[Ca^{2+}]_i$ is elevated under resting conditions in HD.

In vivo models of HD have been shown to exhibit lower total brain volumes and fewer NPCs in neurogenic areas

than controls [75–78]. Accordingly, our data showed an enhanced neurodifferentiation process in HD cells compared with in WT cells, and this process resembled enhanced early stages of cell commitment during neurodevelopment. Altogether, these data suggest that the P2Y2 receptor is a modulator of both neuronal GABAergic commitment and survival and provides an HD therapeutic target given its role in controlling spontaneous $[Ca^{2+}]_i$ oscillations (Supplementary Fig. 8).

Compliance with ethical standards

Conflict of interest HU receives consulting fees and support from TissueGnostics GmbH, Vienna, Austria.

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4.2.7. Hyperactivation of P2X7 receptors as a culprit of COVID-19 neuropathology. *Molecular Psychiatry*, 2021.

REVIEW ARTICLE

Hyperactivation of P2X7 receptors as a culprit of COVID-19 neuropathology

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Abstract

Scientists and health professionals are exhaustively trying to contain the coronavirus disease 2019 (COVID-19) pandemic by elucidating viral invasion mechanisms, possible drugs to prevent viral infection/replication, and health cares to minimize individual exposure. Although neurological symptoms are being reported worldwide, neural acute and long-term consequences of SARS-CoV-2 are still unknown. COVID-19 complications are associated with exacerbated immunoin-flammatory responses to SARS-CoV-2 invasion. In this scenario, pro-inflammatory factors are intensely released into the bloodstream, causing the so-called "cytokine storm". Both pro-inflammatory factors and viruses may cross the blood–brain barrier and enter the central nervous system, activating neuroinflammatory responses accompanied by hemorrhagic lesions and neuronal impairment, which are largely described processes in psychiatric disorders and neurodegenerative diseases. Therefore, SARS-CoV-2 infection could trigger and/or worse brain diseases. Moreover, patients with central nervous system disorders associated to neuroimmune activation (e.g. depression, Parkinson's and Alzheimer's disease) may present increased susceptibility to SARS-CoV-2 infection and/or achieve severe conditions. Elevated levels of extracellular ATP induced by SARS-CoV-2 infection may trigger hyperactivation of P2X7 receptors leading to NLRP3 inflammasome sti-mulation as a key mediator of neuroinvasion and consequent neuroinflammatory processes, as observed in psychiatric disorders and neurodegenerative diseases. In this context, P2X7 receptor antagonism could be a promising strategy to prevent or treat neurological complications in COVID-19 patients.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first identified in December 2019 as the infectious agent of coronavirus disease 2019 (COVID-19).

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SARS-CoV-2 invades cells using prominent spike protein, which binds to cellular membrane receptors. Host cell receptors recognized by SARS-CoV-2 spike proteins include angiotensin-converting enzyme 2 (ACE2) [1, 2] and CD147 (basigin) [3, 4] besides involving virus spike protein priming/processing by transmembrane serine pro-tease 2 (TMPRSS2) [5]. SARS-CoV-2 enters the cell through receptor-mediated endocytosis or receptor-independent entry, as shown for HEK293/hACE2 cells [6]. The challenging question is, whether the infection can occur through extracellular microvesicles shed from infected cells. Such mechanism is often called "trojan horse" and has been proposed for human immunodefi-ciency virus (HIV) [7, 8]. Although neural acute and long-term consequences of SARS-CoV-2 infection are still unknown, neurological symptoms are being reported worldwide. Therefore, urgent challenges are to identify, ameliorate, or even eliminate these effects.

COVID-19 complications are widely associated with exacerbated immunoinflammatory responses upon SARS-CoV-2 lung invasion. In this scenario, pro-inflammatory factors are intensely released, the so-called "cytokine storm", which enter the bloodstream, and reach other organs (Fig. 1A) [9]. In this sense, both pro-inflammatory factors and viruses can cross the blood–brain barrier (BBB) and enter the central nervous system (CNS), initiating a neuroinflammatory process [10]. BBB permeability is increased in patients with neurodegenerative diseases, which could facilitate SARS-CoV-2 neuroinvasion. In addition, the neuroinflammatory insult could increase susceptibility to neurodegeneration in patients who are not yet suffering from these diseases. While confirmation of this hypothesis may take years, possible long-term consequences of COVID-19 need to be highlighted.

Neurotropism of human coronaviruses has already been demonstrated in small animals and in autoptic studies of brains infected with the severe acute respiratory syndrome coronavirus (SARS-CoV) [11], which was responsible for the severe acute respiratory syndrome (SARS) outbreak during 2002–2003. Image studies [12, 13] and postmortem examination [14] of the brain of COVID-19 patients also revealed hemorrhagic lesions accompanied by neuronal injury and neuroinflammation signals. These brain pathologies are described as both etiological factors and consequences of psychiatric disorders and neurodegenerative diseases [15]. Therefore, it is plausible to suggest that patients with COVID-19 are prone to develop neurological disorders, as well as patients suffering from these conditions are more susceptible to SARS-CoV-2 infection. In this context, understanding the mechanisms underlying
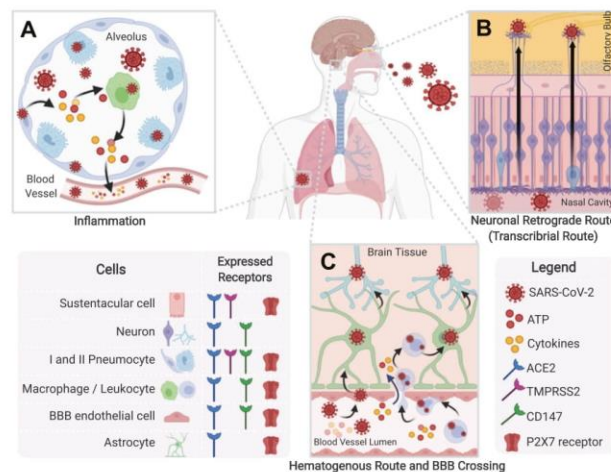

Fig. 1 Possible routes of SARS-CoV-2 infection of the central nervous system and cytokine storm involvement. A In the lung, SARS-CoV-2 may infect type I and II pneumocytes (dark and light blue, respectively) and proliferate in these cells. SARS-CoV-2 invades type I pneumocytes following binding to TMPRSS2 and possibly CD147 receptors, whereas invasion of type II pneumocytes additionally involves binding to ACE2 [171, 172]. The distressed cells release pro-inflammatory factors and DAMPs, such as ATP, which recruit macrophages (green) by activating P2X7 receptors. These macrophages increase the release of cytokines, chemokines, and ATP, inducing the cytokine storm. ATP could also activate P2X7 receptors in type I pneumocytes. These pro-inflammatory factors, as well as the virus, reach the circulatory system and can induce inflammatory responses in other tissues, in part through the activation of P2X7 receptors by released ATP. B In the neuronal retrograde pathway, the virus infects peripheral neurons and uses synaptic connections to reach the CNS. In the transcribrial pathway, SARS-CoV-2 can multiply in sustentacular cells (pink) of the nasal cavity, using ACE2 and TMPRSS2 receptors for infection or in olfactory sensory neurons (purple) following binding to CD147 receptors. Thus, the virus uses these neurons to reach the CNS olfactory bulb (yellow) and infect cells using ACE2 receptors [173]. Sustentacular cells express P2X7 receptors [174]. C Once in the bloodstream, the virus can reach and infect blood–brain barrier (BBB) endothelial cells (pink), through binding to ACE2 and CD147 [175], and perivascular astrocytes (green), through binding to ACE2 [172]. In COVID-19, the BBB shows increased permeability due to the inflammatory process and death of endothelial cells and astrocytes, possibly resulting from P2X7 receptor activation. Although not yet confirmed, the virus could infect monocytes (purple) using CD147 receptors [176], which in turn can pass the BBB. Inside the CNS, the virus can infect neurons (blue) and other neural cell, by binding to ACE2 [173] and possibly to CD147 [177]. Moreover, cytokines present in circulating blood can also reach the CNS through BBB breaches and induce neuroinflammation, sensitizing the brain. Created with BioRender.com.

COVID-19 neuropathology is essential for the development of therapeutic strategies.

Hyperactivation of P2X7 receptors is closely related to inflammatory processes since they are stimulated by ATP released from distressed cells and induce inflammasome activation [16–18]. P2X7 receptors are ATP-gated ion channels widely expressed in the CNS [19], and their activation induced by viral infection leads to molecular (mainly neuroimmune response activation, reactive oxygen species (ROS) formation, and glutamate release) and behavioral alterations [17, 20] as well as to mental disorders [21].

Based on this, we postulate that both, neuroinvasion through the BBB and hyperstimulation of neuroimmune responses observed during COVID-19 infection, are mediated by hyperactivation of the P2X7 receptor, possibly through NLRP3 inflammasome stimulation. This cascade could lead to COVID-19-associated psychiatric disorders and neurodegenerative diseases. Thus, P2X7 receptor antagonism would be a promising strategy to prevent or treat neurological complications in infected patients. This concept will be discussed in detail in our review.

Neurological symptoms in COVID-19

An increasing number of studies reports the manifestation of neurological symptoms in patients with COVID-19. As reviewed by Pezzini and Padovani, neurological manifestations affect between 4.2 and 100% of studied patients: dizziness (prevalent in 7.6–46.1%), headache (5.1–77.1%), impaired consciousness (9.5–64.1%), and seizures (1.2–26%) [22]. Noteworthy, in COVID-19 patients presenting at least one neurological symptom, the prevalence of acute stroke (2.9–76.8%), confusion (14.2–65%), and encephalitis (0–27.9%) stands out. COVID-19 patients also present other nervous system symptoms, such as impaired taste (10–19.2%) and smell (6–21.7%), dysautonomia (4.3–12%), and acute inflammatory demyelinating polyneuropathy (0–16,2%) [22].

Together with an acute cerebrovascular disorder, all symptoms are correlated with the consequences of strokes or micro-strokes [23]. Moreover, reported impaired olfaction could be due to CNS invasion or direct damage of olfactory sensory neurons in the nasal cavity [24].

Psychiatric symptoms are being reported in COVID-19 patients, healthcare workers and in the general population. Table 1 summarizes meta-analysis and systematic reviews on these topics. The studies were selected after searching the PubMed data base on September 20, 2020 for the terms: "(COVID-19 or SARS-CoV-2 or severe acute respiratory syndrome coronavirus 2) and (mental health or psychological health or depression or anxiety or PTSD or PTSS or post-traumatic stress disorder or post-traumatic stress symptoms)".

Although studies on psychiatric symptoms accompanying SARS-CoV-2 illness need further investigation, the available data indicate that COVID-19 patients mainly present depressive symptoms (42–65%), anxiety (37–47%), and PTSS (93–96%). Noteworthy, stress caused by the pandemic situation (e.g., social isolation, fear of infection, and financial instability) may also induce depressive symptoms (14.6–48.3%), anxiety (6.33–50.9%), and PTSS (7–53.8%) in the general population. The stressful situation of healthcare workers, specially the fear to infect relatives, may lead to depressive symptoms (12.2–26.3%), anxiety (13–29%), and PTSS (3–20.7%) as well.

In this scenario, the following proposals are raised and require additional studies: (1) alterations induced by stress exposure (e.g., immune hyperactivation) could both facilitate SARS-CoV-2 infection as well as aggravate COVID-19 symptoms; (2) neurological symptoms are probably not specifically related to SARS-CoV-2 invasion, but they are a general consequence of infectious disorders (which include sickness behavior), stressful events (pandemic situation), and/or immune response hyperactivation ("cytokine storm"); (3) genetic and/or environmental factors affect the development of neurological symptoms, as these responses highly vary across the population. Clarifying these issues would improve the understanding on SARS-CoV-2 infection and direct the search for treatments.

SARS-CoV-2 neuroinvasion

COVID-19 patients are presenting neurological symptoms worldwide. Although brain analysis of these patients is not being widely performed, a study detected SARS-CoV-2 in 8 of 21 postmortem brain tissues, based on reverse transcriptase polymerase chain reaction [25]. Moreover, neuroinvasion capability of SARS-CoV-2 was observed in human brain organoids, especially in neuronal cells, such as neural progenitor and radial glial cells, accompanied by increased cell death [26]. The same study found positive staining for SARS-CoV-2 spike protein in the brain of three COVID-19 patients, with different expression patterns and staining intensities [26]. Neuroinvasion potential of SARS-CoV-2 was demonstrated in mice expressing human ACE2 and correlated with increased mortality independent from respiratory infection [26]. SARS-CoV-2 also seems to invade infants' brains, since immunohistochemical analysis of the postmortem brain of a 1-year-old infant showed positive staining in the choroid plexus, ventricles and cerebral cortex [27]. Remarkably, a case report demonstrated the presence of SARS-CoV-2 viral particles in neural tissue and brain capillary

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Table 1 Summary of systematic reviews and meta-analysis of psychiatric symptoms exhibited by the general population, healthcare workers, COVID-19 patients, and psychiatric patients due to the COVID-19 pandemic outbreak.

Psychiatric outcome	Prevalence % (95% CI)	Total sample size	Sample location	Studies included	Reference
Depression		173,662	China, Iran, Italy, Singapore, Vietnam	50	[178]
Overall	26% (20–33%)				
General population	24% (14–36%)				
Healthcare workers	25% (19–32%)				
COVID-19 patients	42% (28–57%)				
Anxiety					
Overall	26% (21–31%)				
General population	26% (20–32%)				
Healthcare workers	24% (16–32%)				
COVID-19 patients	37% (19–57%)				
Psychological distress					
Overall	34% (27–42%)				
General population	26% (21–32%)				
Healthcare workers	41% (19–65%)				
Stress					
Overall	34% (20–50%)				
General population	36% (5–75%)				
Healthcare workers	33% (19–50%)				
Post-traumatic stress symptoms					
Overall	27% (12–45%)				
General population	15% (4–31%)				
Healthcare workers	13% (11–16%)				
COVID-19 patients	96% (95–97%)				
Poor sleep quality					
Overall	40% (25–57%)				
General population	34% (12–60%)				
Healthcare workers	43% (28–59%)				
COVID-19 patients	82% (66–92%)				
Insomnia					
Overall	30% (12–52%)				
General population	7% (7–8%)				
Healthcare workers	37% (32–42%)				
Depression		93,569	China, Denmark, Nepal, Spain, Turkey, USA	19	[179]
General population	14.6–48.3%				
Anxiety					
General population	6.33–50.9%				
Post-traumatic stress disorder					
General population	7–53.8%				
Psychological distress					
General population	34.43–38%				
Stress					
General population	8.1–81.9%				
Depression		17,330	China	8	[180]
Healthcare workers vs. professionals from other areas	12.2% vs. 9.5 % (OR = 1.3246; 95% CI 1.0930–1.6053)				
Anxiety					
Healthcare workers vs. professionals from other areas	13.0% vs. 8.5% (OR = 1.6152; 95% CI 1.3283–1.9641)				
Stress		63,439	China, India, Iran, Iraq, Italy, Japan, Nepal, Nigeria, Spain, UK	17	[181]
General population	29.6% (24.3–35.4%)				
Asia	27.9% (19.7–37.8%)				
Europe	31.9% (23.1–42.2%)				
Anxiety					
General population	31.9% (27.5–36.7%)				
Asia	32.9% (28.2–37.9%)				
Europe	23.8% (16.2–33.5%)				
Depression					
General population	33.7% (27.5–40.6%)				
Asia	35.3% (27.3–44.1%)				

Table 1 (continued)

Psychiatric outcome	Prevalence % (95% CI)	Total sample size	Sample location	Studies included	Reference
Europe	32.4% (21.6–45.5%)				
Psychological distress		N.R.	N.R.	40	[182]
Healthcare workers exposed to SARS/MERS/COVID-19	37.8% (28.4–48.2%)				
Burnout					
Healthcare workers exposed to SARS/MERS/COVID-19	34.4% (19.3–53.5%)				
Anxiety					
Healthcare workers exposed to SARS/MERS/COVID-19	29.0% (14.2–50.3%)				
Depressive symptoms					
Healthcare workers exposed to SARS/MERS/COVID-19	26.3% (12.5–47.1%)				
Post-traumatic stress disorder					
Healthcare workers exposed to SARS/MERS/COVID-19	20.7% (13.2–31%)				
Anxiety		162,639	Argentina, Brazil, Chile, China, Denmark, Greece, India, Iran, Israel, Italy, Japan, Mexico, Pakistan, Singapore, Spain, Turkey, Vietnam	62	[183]
Overall	33% (28–38%)				
General population	32% (25–39%)				
General population—Italy	81% (80–83%)				
Healthcare workers	26% (18–34%)				
Healthcare workers—Singapore	7% (5–9%)				
Healthcare workers—Italy	57% (52–63%)				
Psychiatric patients with moderate-to-severe anxiety	24% (14–33%)				
COVID-19 patients	47% (34–61%)				
COVID-19 patients with type 2 diabetes—India	40% (30–50%)				
COVID-19 patients with Parkinson's Disease—Iran	82% (74–88%)				
Depression					
Overall	28% (23–32%)				
General population	27% (22–33%)				
General population—Italy	67% (65–69%)				
Healthcare workers	25% (17–33%)				
Healthcare workers—Singapore	9% (7–12%)				
Healthcare workers—China	51% (48–53%)				
Psychiatric patients with moderate-to-severe depression	22% (13–32%)				
COVID-19 patients—China	65% (51–77%)				
Distress					
Overall	35% (23–47%)				
Stress					
Overall	40% (20–60%)				
Insomnia					
Overall	32% (25–39%)				
Post-traumatic stress symptoms/disorders					
General population	16% (15–17%)				
Healthcare workers	3% (2–4%)				
COVID-19 patients	93% (92–95%)				
Anxiety		33,062	China, Singapore	12	[184]
Healthcare workers	23.21% (17.77–29.13%)				
Male	20.92% (11.86–31.65%)				
Female	29.06% (20.21–38.78%)				
Depression					
Healthcare workers	22.93% (13.16–34.38%)				
Male	20.34% (11.57–30.75%)				
Female	26.87% (15.39–40.09%)				
Insomnia					
Healthcare workers	34.32% (27.45–41.54%)				

CI Confidence interval, *OR* Odds ratio, *N.R.* Not reported

endothelium of a Parkinson's disease patient, which was associated with the worsening of neurological symptoms [28].

However, the mechanism of SARS-CoV-2 infection of the brain is still unknown. Invasion routes of the CNS by other viruses include: (a) the hematogenous route, in which

viruses use the bloodstream to reach and invade epithelial cells from the BBB or the blood–cerebrospinal fluid barrier, or use leukocytes as a vector to enter the CNS; (b) the neuronal retrograde route, in which viruses invade peripheral neurons and reach CNS, including the transcribrial route, using olfactory sensory neurons in the nasal cavity (Fig. 1B) [10].

In the hematogenous route, the virus must be capable of crossing the BBB (Fig. 1C). This barrier is composed of endothelial cells, pericytes, and astrocytes. The restricted permeability of the BBB is a reflex of the connection between brain microvascular endothelial cells and tight cell–cell junctions. The BBB is disrupted under inflammatory conditions [29, 30]. In the "Trojan horse" mechanism of CNS invasion, infected leukocytes pass the BBB. This mechanism is observed for HIV, and since SARS-CoV can infect immune cells, it is likely that SARS-CoV-2 also uses this route toward the CNS [10]. Viral infection also affects BBB integrity by different mechanisms, including phosphorylation of tight junction proteins, disruption of the basal lamina or of the actin cytoskeleton, or by invading BBB-epithelial cells and furthermore astrocytes [10, 31].

Evidence of CNS invasion through neuronal retrograde routes was reported for coronaviruses, such as HCoV-OC43, HEV67 and avian bronchitis virus [32]. Once respiratory and digestive tracts of animals were infected, coronaviruses invaded peripheral neurons and passed through synaptic connections until they reached medullary neurons and subsequently other neurons and glial cells of the CNS [32]. As the main entry route of SARS-CoV-2 in humans, cells from the nasal cavity could be susceptible to viral infection and replication. Studies reported that human olfactory sustentacular cells express both ACE2 and TMPRSS2 virus receptors [33]. Although current literature data reports that human olfactory sensory neurons do not express these proteins, they express CD147 that could allow SARS-CoV-2 neuroinvasion (Fig. 1) [33, 34]. In fact, SARS-CoV, MERS-CoV, and HCoV-OCR43 were able to invade the murine CNS using the transcribrial route, infecting olfactory sensory neurons of nasal cavity, and passing to other neural cells, indicating that SARS-CoV-2 could use the same mechanism [24, 35–37].

Inflammation and CNS-related lethality of COVID-19

Severe COVID-19 patients commonly develop the Acute Respiratory Distress Syndrome [38], which is characterized by inflammatory injury to the alveoli–capillary membrane, leading to lung over-permeability and increased pulmonary edema fluid into the airspaces, resulting in the lack of respiratory capacity [39]. This overreaction of the innate immune system against viral infection induces the so-called "cytokine storm", comprising of: (1) the release of large

amounts of several pro-inflammatory cytokines (interferons IFNα and IFNγ, interleukins [IL-1β, IL-6, IL-12, IL-18 and IL-33], tumor necrosis factor [TNF]-α, and transforming growth factor [TGF]-β) and chemokines (CXCL10, CXCL8, CXCL9, CCL2, CCL3, CCL5); (2) release of renin–angiotensin aldosterone system (RAAS) mediators and increasing blood levels of angiotensin II (Ang II); and lately (3) amplification of the innate immune system response and activation of its major humoral arm, the complement cascade (ComC) [40].

Novel evidence indicates that COVID-19-released mediators merge on a common pathway, upregulating cytosolic danger sensing pattern recognition receptor, which is part of a multiprotein complex of the innate immune system that is called inflammasome, and recognizes both pathogen-associated molecular patterns and self-derived danger-associated molecular patterns (DAMPs) or alarmines [41].

Importantly, upon inflammasome protein assembly and activation, pro-caspase 1 protein is cleaved to functional caspase 1, whose main function is the conversion of the inactive and intracellularly stored pro-inflammatory cytokines, pro-IL-1β and pro-IL-18, into their active forms that are released from cells. This release is facilitated by creating gasdermin D (GSDMD) pore channels in cell membranes. In addition to these two cytokines, gasdermin D channels also mediate the release of several biologically active DAMPs or alarmines, including extracellular ATP, high mobility group protein B1, and S100 calcium-binding proteins A8 and A9 (S1008/9a) [42].

There are various inflammasome subtypes. The NLRP3 inflammasome protein complex is usually involved in virus infections and consists of NLRP3 protein, apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1, and remains in the cytosol in steady-state conditions in an inactive form. Upon activation, it becomes a multiprotein aggregate composed of several NLRP3 molecules (speck complexes), each containing NLRP3 protein, ASC, and pro-caspase 1 [41].

We consider the NLRP3 inflammasome as a trigger of the cytokine storm, as seen in COVID-19 patients, that may be induced by P2X7 receptor activation, including in the brain (Fig. 2). Once SARS-CoV-2 spike protein interacts with ACE2, macrophages/microglia cells can potentiate the immune response through the cleavage of fragments complement component 3a and 5a (C3a and C5a, respectively) and non-lytic C5b-C9 membrane attack complex by ComC, thus activating the NLRP3 inflammasome [41]. Moreover, NLRP3 inflammasome activation during COVID-19 infection is usually triggered by Ang II, which binds to the angiotensin type 1 receptor (AT₁R), leading to vessel contraction and increasing blood pressure. In normal conditions, Ang II is converted by the ACE2 receptor into angiotensin 1–7 (Ang 1–7) [43]. Ang II activates the AT₁R,
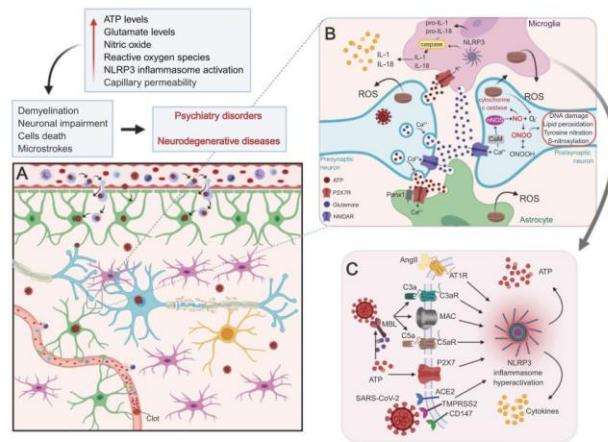

Fig. 2 P2X7 receptor-mediated neuroinflammatory implications of SARS-COV2 invasion in the CNS. A SARS-CoV-2 may alter brain function by reaching the central nervous system (described in Fig. 1) and/or through the cytokine storm-mediated effects. The result is a neuroinflammatory process characterized by microglia (pink) hyperactivation, astrocyte (green) stimulation, and demyelination (yellow caps) of neurons (blue). In addition, the cytokine storm induces blood-clot formation and increased capillary (red) permeability resulting in embolic and hemorrhagic strokes, respectively. **B** In a molecular view, the distressed cells release pro-inflammatory cytokines (yellow circles) and ATP (red circles). ATP activates P2X7 receptors (red) expressed mainly in microglia (pink) and astrocytes (green) resulting in increased Ca^{2+} influx and glutamate (purple circles) release. Glutamate activates NMDA receptors expressed in nerve terminals (blue), which enable Ca^{2+}-dependent exocytosis of ATP and more glutamate release. In this way, an auto regenerative loop is formed causing a massive release of these neurotransmitters augmenting excitotoxicity and cell death. In the postsynaptic neuron (blue), increased $[Ca^{2+}]_i$ leads to Ca^{2+}-cal-modulin (CaM) complex formation and consequent nNOS activation. NO production mediates neurotoxicity via several mechanisms. NO interacts with the iron–sulfur centers in the mitochondrial electron transport chain impairing cellular energy production. NO also produces reactive nitrogen species and reactive oxygen species (ROS). Reaction of NO and superoxide ion (O_2^-, formed by nNOS under low arginine concentrations) generates peroxynitrite (ONOO⁻) and peroxynitrous acid (ONOOH). These free radicals can also decompose into other reactive species, such as hydroxyl radical and peroxides.

Oxidative stress from free radicals includes DNA damage, lipid per-oxidation, tyrosine nitration, and excess S-nitrosylation. These structural changes can lead to protein misfolding and aggregation causing neuronal impairment and/or death. In microglia, K^+ efflux mediated by P2X7 receptor activation may trigger NLRP3 inflammasome assembly and activation through NIMA-related serine/threonine kinase 7 (Nek7) binding. NLRP3 inflammasome mediates the activation of caspase-1, which induces the maturation of interleukins (IL) by cleaving pro-IL-1β and pro-IL-18 in IL-1β and IL-18, respectively. The mature forms of cytokines are secreted worsening the neuroinflammatory process established. **C** The hyperactivation of NPLR3 inflammasome and consequent release of cytokines and ATP can occur by different routes. (I) Activation of the renin–angiotensin system (RAS) leads to elevated levels of angiotensin II (Ang II) that binds to the AT_1R receptor. (II) The N proteins of the SARS-CoV-2 virus activate ComC in a mannan binding lectin (MBL)-dependent manner, producing C3a and C5a anaphylatoxins and forming the non-lytic C5b/C9 membrane attack complex (MAC). ATP can also activate MBL and induce this response. (III) P2X7 receptor activation by ATP induces K^+ influx and inflammasome activation. (IV) SARS-CoV-2 invasion through ACE2, TMPRSS2 or CD147 activates the inflammasome in target cells. Hyperactivation of these pathways leads to activation of caspase 1, release of mature IL-1β and IL-18, the insertion of gasdermin D channels in the cell membrane and the release of danger-associated molecular pattern molecules (DAMPs), which amplify the innate immune response and may lead to cell death by pyroptosis. Created with BioRender.com.

inducing fibrosis, increased ROS release, vasoconstriction, and gut dysbiosis. In contrast, Ang 1–7 binds to Mas receptors (MasR), thus protecting against fibrotic formation, presenting antioxidant and vasodilatory effects. The problem with the conversion of Ang II into Ang 1–7 is that the ACE2 enzyme expressed on surface of cells is a COVID-19 receptor and is blocked or downregulated after binding of virus or even shed from the cell surface [43]. Extracellular ATP can also robustly trigger activation of the NLRP3 inflammasome through P2X7 receptor activation. In addition, ATP is also released into the extracellular milieu upon NLRP3 inflammasome activation, promoting a strong positive feedback loop [44].

SARS-CoV-2-induced systemic inflammatory responses may also result in endothelial damage and consequently increased production of thrombin by inflammatory cytokines during sepsis in a bidirectional way, because inflammation activates coagulation, and coagulation augments inflammatory activity [45]. In this scenario, active inflammatory cells expressing specific protease activated receptors

(PAR1–4) bind to thrombin, while the Toll-like receptor 4 interacts with fibrin. Supporting these events, patients in particular at younger age are often diagnosed with stroke and cardiovascular complications.

Recent evidence indicates that the occurrence of psychiatric disorders in patients is linked to "sterile" inflammation of the brain that may be initiated locally by some stressors affecting nervous tissue or occurs due to a systemic inflammation process [17, 46]. This is supported by the observation that several inflammatory mediators and markers are detected in the peripheral blood of patients with psychiatric and neurodegenerative disorders [17, 46], which could worsen prognoses for COVID-19 outcome. Moreover, clinical data describe correlations between systemic chronic inflammatory processes and psychiatric disorders. This may also explain, why some reported anti-inflammatory treatment strategies ameliorate neurodegeneration [17, 46]. In agreement, pathological increase of AngII-AT$_1$R-mediated activation of NLRP3 inflammasome may initiate psychosis. We believe that bioactive inflammatory mediators released during the cytokine storm, such as extracellular ATP, affect the CNS and may lead to its impairment, mainly through P2X7 receptor activation, as we discuss in the following.

Probable P2X7 receptor roles in COVID-19 processes

The P2X7 receptor is widely expressed through the body [47], including in immune [48], lung [49], and CNS cells, mainly microglia and oligodendrocytes [50–52], whereas its expression in neurons and astrocytes is still under discussion [50, 51, 53–60]. During the progress of infection, ATP release may result from the NLRP3 inflammasome response to COVID-19 infection (i) after virus spike protein interaction with the virus entry receptor ACE2, (ii) due to elevated level of Ang II observed in infected patients, or (iii) in response to activated ComC mediators [61]. Consequently, P2X7 receptors are activated, increasing inflammatory responses as well as modulating RAAS-related pathways and BBB permeability, as we discuss in the following.

P2X7 receptor role in inflammation

The P2X7 receptor is a trimeric ionotropic receptor that belongs to the P2X family of purinergic receptors, presenting low affinity for ATP (EC$_{50}$ around 0.3–1.8 mM) and, under prolonged stimulation, can form pores allowing the passage of large hydrophilic molecules [62–64]. Activation of P2X7 receptor elicits rapid K$^+$ efflux as well as Ca^{2+} and Na$^+$ influx, resulting in the stimulation of several intracellular intermediators, as PLC, PLA2, PKC, MAPK,

PI3K, ERK1/2 and p38, among others [65]. Consequently, P2X7 receptor activation is associated with numerous cellular functions, as plasma membrane blebbing, phosphatidylserine exposure in the membrane, formation of ROS, interleukin secretion, cell death and proliferation [65, 66].

ATP concentration is present in the nanomolar range in extracellular space of healthy tissues [67]. Conversely, in a disease state as infection or brain disease, extracellular ATP levels largely increase and activate P2X7 receptors [68–70]. Consequently, pore formation results in enhanced release of ATP and establishes a positive feedback in pathological conditions. In these circumstances, ATP may act as DAMP, which activates the nuclear transcription factor NF-κB resulting in expression upregulation of pro-IL-1β and pro-IL-18 and the NLRP3 protein [16, 71]. In addition, P2X7 receptor activation triggers K$^+$ efflux, a signal required for efficient NLRP3 inflammasome stimulation [72]. Consequently, there is an activation of caspase-1 and maturation of IL-1β and IL-18, resulting in pro-inflammatory cytokine release [16, 20, 52, 71, 73]. The P2X7 receptor is a major activator of the NLRP3 inflammasome in several cell types [16], including macrophages and microglial cells [72, 74–76]. Moreover, P2X7 receptor activation also promotes the release of other inflammatory mediators such as IL-6, TNF-α, CCL2, CCL3, and CXCL2 [77–80].

In fact, the role of P2X7 receptor as a modulator of inflammatory response is well-established [16, 70]. It was already reported its capacity to modulate acute and chronic infection [81, 82], inflammatory diseases [82], sepsis [83, 84], neuropathic pain [85], and T-cell activation [86]. In addition, P2X7 receptor expression deletion appears to be beneficial in case of acute lung injury, asthma, lung inflammation, and fibrosis [87–91]. Remarkably, P2X7 receptor activation increases neuroinflammatory responses, which have been associated with neurodegenerative diseases, psychiatric disorders and stroke [21, 92–95], as further discussed.

Cytokine storms have been reported in several viral infections [96], including SARS-CoV-2 [9, 97]. Patients infected with SARS-CoV-2 present elevated levels of IL-6, IL-10, IL-1β, INF-γ, CCL2, TNF-α, CXCL10, CCL7, IL-1 receptor antagonist and IL-2 receptor, supposedly associated with disease severity [9, 98–105]. Considering the P2X7 receptor role in inflammation, its activation may be involved in the cytokine storm observed in COVID-19 by stimulating NLRP3 inflammasome, overshooting inflammation with extensive cytokine release, affecting coagulation and leading to diffuse lung edema and infiltration by immune cells and inflammatory cytokines [106]. As discussed by Di Virgilio et al. [106], many processes associated with lung impairment, as seen in COVID-19 patients, are mediated by P2X7 receptors [16]. The massive ATP release following lung mononuclear phagocytes

invasion by SARS-CoV-2 can activate P2X7 receptors of antigen-presenting cells and macrophages, increasing cytokine, chemokine and ATP secretion [16]. In addition, P2X7 receptor knockout mice submitted to a model of lung fibrosis revealed reduced infiltration of inflammatory cells, cytokine release, apoptosis, and fibrosis, while P2X7 receptor agonists increased these deleterious processes [88, 107].

Therefore, we hypothesize that the P2X7 receptor activation is involved in the cytokine storm and associated lung and brain inflammation caused by SARS-CoV-2 infection.

P2X7 receptors and the renin–angiotensin system

The P2X7 receptor, as a central player in inflammation, participates in viral infections. Exerting mostly pro-inflammatory roles [20], the P2X7 receptor is important for pathogen elimination [108], but on the other hand, its activation may be detrimental to the host due to the induction of exacerbated inflammatory responses. Protective roles of P2X7 receptor activation have been described, for instance, against vesicular stomatitis virus, Newcastle disease virus, murine leukemia virus and HSV virus infections [108]. Controversially to the above-cited beneficial P2X7 receptor-mediated viral elimination, this receptor was described to even facilitate virion release from HIV-infected macrophages in an exocytosis-dependent way [109]. Moreover, P2X7 receptor activation exacerbated inflammation, enhanced tissue damage, increased mortality, and worsened lung pathology involving P2X7 receptor expression or activation, such as observed in several other studies with e.g., influenza A virus [110] and adenovirus [111].

Importantly, the P2X7 receptor triggers pathways related to the functioning of the RAAS, importantly implicated in COVID-19 pathology. RAAS mediates key events of the disease, including viral entry in the cell [112, 113], inflammation, and lung fibrosis [114]. The pressor axis of RAAS bases on Ang II actions through the activation of AT$_1$R. ACE is responsible for the cleavage of angiotensin I into Ang II, and Ang II levels are counterbalanced by the depressor axis that degrades Ang II into Ang 1,7 through ACE2 activity. Finally, Ang 1,7 activates MasR [115]. ACE2, independent from its enzymatic activity, serves as SARS-CoV-2 entry route in human cells, but as this enzyme is internalized following virus binding, its enzyme function is lost [6, 116]. Consequently, Ang 1,7 that would exert beneficial effects against CNS damage and neurological deficits [115] is not formed, prevailing Ang II actions mediated by the AT$_1$R promoting fibrosis, lung injury, and importantly neuroinflammation [115].

In fact, the balance between the pressor and depressor axis of RAAS is implicated in neurodegenerative and psychiatric disorders, as carefully reviewed elsewhere

[115, 117]. Briefly, components of the depressor axis correlate to enhanced cognition and cell survival in the brain, orchestrating antioxidant, and anti-inflammatory responses. In agreement, inhibition of the pressor axis attenuates cognitive deficits observed in aging, Alzheimer's disease, Parkinson's disease, vascular cognitive impairment and poststroke cognitive impairment [117].

Since the P2X7 receptor is also involved in lung damage [87–89], neurodegenerative and psychiatric disorders [92, 95, 118], the hypothesis is reasonable that inhibition of this receptor might decrease pathological traits mediated by the RAAS pressor axis and protect against brain and lung injury in COVID-19 patients. Indeed, evidence demonstrates that P2X7 receptor blockade prevents Ang II-triggered pro-inflammatory responses [119]. Furthermore, in rats with diabetic nephropathy, P2X7 receptor expression decreased ACE activity and Ang II levels [120], which might increase ACE2 expression [43] and counteract SARS-CoV-2-induced inflammatory exacerbation.

Conciliating all these findings, we highlight that the blockade of P2X7 receptors might prevent inflammatory exacerbation both, through its direct actions in inflammasome assembly and through RAAS modulation, avoiding deleterious actions of RAAS in several tissues, including the lung and the brain.

P2X7 receptors in BBB permeability

The BBB protects the nervous tissue from direct contact to the blood. Endothelial cells comprise the BBB and are highly selective in consequently filtering the content that is available to neurons [121]. Endothelial cells express high levels of ACE2, which is the main mediator of SARS-CoV-2 infection. The infection and death of these cells may disrupt the barrier and let both the virus and inflammation molecules to access the nervous system (Fig. 1C).

As previously mentioned, ATP acts as a DAMP and activates the P2X7 receptor, which is largely expressed by endothelial cells and astrocytes/microglial cells, releasing pro-inflammatory cytokines and amplifying the immune response [122]. The opening of P2X7 receptor channels leads to IL-1β production mainly through NLRP3 inflammasome activation [123], thus contributing to the disruption of the BBB. In this way, SARS-CoV-2 infection can disrupt the BBB by directly infecting and killing endothelial cells, as well as by triggering P2X7 receptor signaling. In agreement, P2X7 receptor antagonism protects against BBB disruption during intracerebral hemorrhage [124]. Moreover, disruption of the BBB due to other viral infections has already been proven to trigger long-term development of neurological disorders, such as Alzheimer's disease, depression, anxiety and multiple sclerosis [125–127].

Both intracerebral hemorrhage, against which the P2X7 receptor antagonist protects [124], and clots are responsible for the primary stroke in the brain [128]. COVID-19 patients are suffering acute cerebrovascular disorder and other correlated neurological symptoms as consequences of stroke or micro-strokes [24]. Stroke includes medical conditions that affect blood vessels of the brain, impairing cerebral circulation as consequence of damaged or deformed arteries (Fig. 2A). Diverse hospitals reported increasing levels of patients affected by stroke during SARS-CoV-2 infection [129, 130]. Klok and colleagues observed disturbing increases in the incidence of thrombotic complications in critically ill intensive care unit patients as high as 31% and recommended pharmacological prophylaxis against thrombosis [24].

The most recent hypothesis for the increased levels of strokes in COVID-19 patients is that hundreds of clots produced in the lungs due to excessive inflammation process may be transported into brain arteries, causing stroke by ischemia. Further, the virus can access the brain through the blood stream and infect BBB cells, as observed for other viruses [131]. Both ways can lead to increased inflammation due to hypoxia, increased acidification and release of DAMPs at the niche of the injury, disrupting the BBB and damaging the CNS and, thus, leading to the development of neuropsychiatric disorders.

Neuropsychiatry and neurodegenerative disorders associated to COVID-19

As already mentioned, inflammation is mediated by cytokines, chemokines, ROS and other bioactive molecules. These molecules act like DAMPs, signaling astro- and microgliosis. The first immune response to an insult in the nervous system is the activation of microglial cells of the M2 phenotype, which is involved in anti-inflammatory mechanisms for defense and injury repair [132]. However, the sustained activation of these cells induces the phenotypic change to the M1 type, involved in pro-apoptotic processes, production and release of cytokines and ROS that could induce neurodegeneration. In fact, neuroinflammation is closely related to neurodegenerative diseases, as Huntington's [133], Alzheimer's [134] and Parkinson's disease [135] and neuropsychiatric disorders, such as major depressive disorder, bipolar disorder and anxiety [136]. Since the CNS immune response is also modulated by peripheral components [137], we hypothesized that the cytokine storm induced by viral infection could stimulate neuroinflammation and neuronal death, facilitating the development of these diseases (Fig. 2B). Pathological inflammation may be induced by P2X7 receptor activation, and its antagonism has been proposed as a therapeutic approach for COVID-19 treatment [106].

The role of the P2X7 receptor in neuroinflammatory processes in neuropsychiatric and neurodegenerative diseases is widely studied [95, 118]. In neurodegenerative diseases, cell death is accompanied by neuroinflammatory processes and massive ATP release [138]. In animal models for neurodegeneration P2X7 receptor antagonism exerted beneficial anti-inflammatory effects [138, 139], indicating that P2X7 receptor activation and subsequent neuroinflammation is tightly related to neurodegeneration worsening. Experimental evidence supports that viral infection may evoke neuronal death, encephalopathy, myelin destruction and juvenile Parkinson's disease development; however, these studies did not include coronaviruses [140]. These evidences indicate that viral infection facilitates neurodegenerative disease development, as Alzheimer's disease, Multiple Sclerosis and Parkinson's Disease [140]. In fact, murine coronavirus is used to induce encephalitis in mice with prominent demyelination and axonal damage. Based on observed pathophysiology and disease progression, this has been proposed as an animal model of multiple sclerosis [141].

Curiously, anosmia is one of the neurological symptoms reported by SARS-CoV-2 patients. It is worthwhile noting that olfactory impairment is an early predictor of Parkinson's disease development due to α-synuclein aggregation in the olfactory bulb [142]. Although these symptoms might not be disease-related, studies show that α-synuclein induces mitochondrial dysfunction and microglial activation caused by P2X7 receptor stimulation [143, 144]. Further investigation of neural cell marker expression of COVID-19 patients could clarify, whether neuroinflammation is related to the observed anosmia.

Neuroinflammation and increased levels of ATP are observed in neuropsychiatry disorders, supposedly correlated with P2X7 receptor activation [118, 145]. Corroborating this hypothesis, antidepressant treatment decreased P2X7 receptor expression in the ventral hippocampus of stressed animals [146]. Moreover, the treatment with a selective P2X7 receptor antagonist (A-804598) induced antidepressant-like effect associated with inflammasome stimulation [147] and BDNF signaling activation [148] in rodent hippocampus. In this context, we propose that the cytokine storm and cell death induced by SARS-CoV-2 infection results in P2X7 receptor-mediated neuroinflammation, leading to the development of neuropsychiatry disorders. Noteworthy, the P2X7 receptor antagonist JNJ-54175446 is undergoing clinical trials with treatment-resistant depressive patients (EudraCT number 2018-001884-21 at www.clinicaltrialsregister.eu).

As mentioned, a study with 714 patients with COVID-19 found a prevalence of self-reported post-traumatic stress disorder symptoms in 96.2% of them [149]. Another study with 114 patients reported that 34.7% of patients presented anxiety symptoms, while 28.5% of them displayed

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depression symptoms [150]. Moreover, COVID-19 related psychiatric symptoms (including anger, anxiety, suicidal ideas, hallucinations, insomnia, impaired memory, poor concentration, time disorientation, fear/panic, pressured speech, mood alterations, pessimistic thinking, crying spell and persecutory ideas) have been largely described during and after the occurrence of other respiratory pandemics, such as MERS [151] and SARS [152–159]. Further indication for psychiatric implications of coronaviruses infections came from a study of Okusaga et al. [160], who connected seropositivity for human coronavirus strain L63 with mood disorders and suicide attempts. The study could come to a limited conclusion, as it would need to be conducted with more control and coronavirus-serum positive volunteers. However, the authors of this study distinguished between two possible scenarios, which might be important for the understanding of neuronal effects of COVID-19: (1) the connection between depression and immune responses with possible pro-inflammatory interleukin overshooting, which may trigger oxidative stress and neuroinflammation as part of the causes of psychiatric disorders; and (2) viral infection acting as a form of stress. Dysregulated stress and neuroinflammatory responses might cause hypothalamic–pituitary–adrenal (HPA) axis dysfunction. Alterations in the HPA axis have been linked to mood diseases, and HPA activation has been connected with suicide. Hiroi et al. found hyperplastic adrenals in suicide victims, corroborating with such hypothesis [161]. In line, a small COVID-19 patients' study concluded that preexisting psychiatric disease patients reported mental symptom worsening following infection with the virus [162].

Noteworthy, gene knockout or pharmacological inhibition of P2X7 receptors induced antidepressant-like behavior in mice exposed to stress, accompanied by HPA axis restoration [163, 164]. This axis is also activated for counteracting tissue damage, evoked by cytokine storms, in SARS-CoV-2 infection, as previously reviewed [165]. Thus, the HPA axis might provide a connection between coronaviruses and psychiatric disorders. Noteworthy, such mechanism would explain the possible propensity for the development of mood disorders triggered by COVID-19 infection as well as raise the hypothesis that mood disorder patients might be more prone to severe COVID-19 disease development. These patients already carry augmented inflammation patterns, such as systemic inflammation, or suffer from a cytokine storm [166] and show enhanced P2X7 receptor expression, as previously discussed. However, a wide variety of responses to SARS-CoV-2 infection are not yet understood. Further studies are needed to elucidate genetic and environmental factors that could affect individual vulnerability to COVID-19 infection.

Finally, increasing VEGF concentrations, which recruit inflammatory cells into the brain and sustain neuroinflammation, have been named as target for COVID-19 treatment [167]. The COVID-19 entry receptor ACE2 activates RAAS for neuroinflammation and VEGF synthesis by Ang II binding to AT_1R (reviewed in ref. [167]). Besides ACE2, aberrant P2X7 receptor activation also induces VEGF release and signaling in the brain [168]. Thus, although VEGF involvement in major depressive disorder is controversially discussed [169, 170], this growth factor could be involved in the connection between COVID-19, major depressive disorder and purinergic signaling.

Conclusion

Several lines of evidence have raised the possibility of neuroinvasion by SARS-CoV-2 (Fig. 1), which may cause short- or long-term impairment of the CNS. The main mechanism involved in this scenario is neuroinflammation, a critical process in psychiatric and neurodegenerative disease development. SARS-CoV-2 infection induces a cytokine storm that could trigger and/or worse neuroinflammatory processes. Patients with mental disorders associated to neuroimmune activation such as depression, Parkinson's or Alzheimer's disease may also present increased susceptibility to SARS-CoV-2 infection and/or severe disease development. Neuroimmune response hyperstimulation observed during viral infection and in mental disorders may be mediated by P2X7 receptor activation (Fig. 2). In view of that, we suggest P2X7 receptor as a key mediator of the neuroinflammatory process as a possible consequence of SARS-CoV-2 infection. In this context, P2X7 receptor antagonism could be a promising strategy to avoid and treat psychiatric disorders and neurodegenerative diseases of COVID-19 patients.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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4.2.8. Cancer metabostemness and metabolic reprogramming via P2X7 receptor.
Cells, 2021.

cells

MDPI

Review

Cancer Metabostemness and Metabolic Reprogramming via P2X7 Receptor

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Abstract: The heterogeneity of tumor cell mass and the plasticity of cancer cell phenotypes in solid tumors allow for the insurgence of resistant and metastatic cells, responsible for cancer patients' clinical management's main challenges. Among several factors that are responsible for increased cancer aggression, metabolic reprogramming is recently emerging as an ultimate cancer hallmark, as it is central for cancer cell survival and self-renewal, metastasis and chemoresistance. The P2X7 receptor, whose expression is upregulated in many solid and hematological malignancies, is also emerging as a good candidate in cancer metabolic reprogramming and the regulation of stem cell proliferation and differentiation. Metabostemness refers to the metabolic reprogramming of cancer cells toward less differentiated (CSCs) cellular states, and we believe that there is a strong correlation between metabostemness and P2X7 receptor functions in oncogenic processes. Here, we summarize important aspects of P2X7 receptor functions in normal and tumor tissues as well as essential aspects of its structure, regulation, pharmacology and its clinical use. Finally, we review current knowledge implicating P2X7 receptor functions in cancer-related molecular pathways, in metabolic reprogramming and in metabostemness.

Keywords: cancer stem cells; stemness; P2X7 receptor; P2X7A; P2X7B; purinergic signaling; metastasis; metabolism; metabolic reprogramming; chemotherapy and chemoresistance

1. Introduction

P2X7 receptor is an ATP-gated purinergic ion channel widely present in different cells and tissues, such as stem cells [1], brain [2], intestine [3], kidney [4] and mostly expressed in immune [5] and several cancer cells [6–8].

Overstimulation of the receptor can activate a large pore in the membrane. This is not an exclusive feature of P2X7 receptor, as it has also been identified in $TRPV_1$, ASICs, P2X2 and P2X4 receptors [9]. Nonetheless, while further receptors open pore upon prolonged stimulation, the P2X7 receptor takes only milliseconds to do the same [9]. The pore opening is responsible for many of its functions, including tumor cell death [10]. In contrast, its tonic activation promotes tumor growth [11]. It has been confirmed that the P2X7 receptor exerts these dual activities in the majority of cancers.

The P2X7 receptor binds its physiological agonist ATP with low affinity. Therefore, its activation is only achieved when the level of ATP is significantly elevated in the extracellular space [7]. Since, ATP concentration is extremely higher in tumor microenvironment compared to healthy tissues, the P2X7 receptor may function as key signal transducer in the communication of TME and cancer cells [12]. In this way, blockade of the P2X7 receptor emerges as a potential effective [9] anticancer treatment.

Although the role of the P2X7 receptor in oncogenesis has not been fully elucidated, the link between functions attributed to this receptor and tumor cells has been broadly

recognized [13]. P2X7 receptor stimulation can contribute to tumor biology in different ways, such as maintenance of cancer stem cells (CSC), tumor progression, chemoresistance and metastasis [14].

The emerging cancer hallmark metabolic reprogramming is a fundamental aspect of cancer metastasis and therapy resistance [15]. P2X7 receptor expression has a pivotal role in metabolic diseases and cancer metabolic reprogramming [16]. The receptor tonic stimulation increases mitochondrial potential ($\Delta\psi$m), the glycolytic rate, glycogen storage and oxidative phosphorylation efficiency. On the other hand, its overstimulation is detrimental for cells, resulting in decreased $\Delta\psi$m, mitochondrial fragmentation and cell death [17–20]. Besides, the absence of the P2X7 receptor reduces mRNA and protein expression of fatty acid metabolism key enzymes, such as FASN and acetyl-CoA carboxylase (ACC), and increases serum triglyceride and cholesterol levels, glucose intolerance and insulin resistance, which decreases stemness, proliferation, survival, invasiveness and therapeutic resistance [21]. This evidences strongly correlate the involvement of the receptor in metabostemness and metabolic reprogramming of cancer cells.

Metabostemness may be referred to as "the metabolic parameters causally controlling or functionally substituting the epitranscriptional orchestration of the genetic reprograming that redirects normal and tumor cells toward less differentiated (CSCs) cellular states" [22]. Here, we summarize important aspects of P2X7 receptor structure, regulation, pharmacology and its clinical use. Moreover, the recent findings connect the P2X7 receptor actions with metabostemness and with metabolic reprogramming of cancer cells.

2. The Purinergic Signaling System—Structural Insights Focusing on the P2X7 Receptor

Purinergic or pyrimidinergic nucleotides and nucleosides present in the extracellular space act as co-transmitters, neuromodulators or ligands in cell signaling events, thus, participating in purinergic signaling [23,24]. The purinergic system is composed of P1, P2 and recently proposed, P0 receptors (P1Rs, P2Rs and P0Rs) [25]. P1Rs are G protein-coupled receptors responsive to adenosine. P2Rs are either ionotropic channels responsive to ATP (P2XRs) or metabotropic/G protein-coupled receptors responsive to ATP, ADP, UTP, UDP or UDP-glucose (P2YRs). Finally, P0Rs are G protein-coupled receptors responsive to adenines [26].

Seven types of P2X subunits are known so far, ranging from P2X1 to P2X7. Each P2XR ion-channel is composed of three subunits, either in homo- or heterotrimeric compositions [27]. Each subunit contains two hydrophobic transmembrane domains (TM1 and TM2) linked by a large, glycosylated and cysteine-rich extracellular loop [28–30]. In the intracellular space, the P2X subunits present a short *N*-terminal domain with approximately 30 amino acid residues [31] and a length-variable C-terminal domain [30] with 30–240 amino acid residues [31]. TM domains participate in channel gating and conductance, and may be involved in the differential desensitization kinetics of P2XR subtypes [31,32]. TM1 is associated chiefly with channel opening and receptor sensitization [33], whereas TM2 internally coats the pore permeable to sodium, potassium and calcium [24,34]. P2XR-intracellular domains have been strongly related to membrane trafficking, homologous desensitization, protein-protein interactions and phospholipids modulation [28,31,35–37]. The P2X7 receptor almost does not desensitize, and sustained ATP stimulation leads to more distinct responses than acute stimulation does [31,32].

The P2XR ectodomain presents three available ATP-binding sites, although it was recently showed that the occupancy of two is enough to activate these receptors [38,39]. The sequential binding of each ATP molecule leads to asymmetrical conformational changes that decrease agonist affinity to the binding site, leading to a negative cooperativity mechanism [40]. Regardless of amino acid residues underlying ATP-binding being highly conserved among different subtypes [31], the P2X7 receptor has the lowest ATP sensitivity within the P2XR family [37].

As response to ATP stimulation, the P2X7 receptor channel opening promotes mono- or divalent cation currents, generally by Na^+ and Ca^{2+} influx and K^+ efflux, leading to

cell depolarization and downstream Ca^{2+} signaling events. Sustained stimulation drives a non-selective long-lasting opening, which allows the permeation of larger molecules of up to 900 Da, including common fluorescent dyes [10,41].

There are several isoforms of the P2X7 receptor, named P2X7A-J. The P2X7 receptor can be assembled by just one of them or by a composition of two or three different isoforms [42]. P2X7 receptor-related functions in physiological or oncological processes are associated with the expression levels of its genetic variants [43,44]. Among them, P2X7A and B isoforms are the most studied in humans. P2X7A has a C-terminal crucial for opening membrane large pores causing apoptosis, while P2X7B has a reduced C-terminal, being incapable of inducing cell death [11], while it is able to promote proliferation of stem and tumor cells [43,44] and participates in the cell differentiation process [44]. Although the role of isoforms for cancer metabolism reprogramming and metabostemness has been clarified yet, we hypothesize that a differential contribution of these isoforms account for cancer cell survival and self-renewal, metastasis and chemoresistance. Understanding the functions of P2X7 isoforms on cancer metabolism may open avenues to more efficient therapy against chemoresistant and metastatic tumor.

3. Metabolic Pathways Driving Cancer Cell Survival and Stemness

Metabolic reprogramming is recognized as a cancer hallmark [45], being interpreted both as a driver of malignant transformation and a consequence of this transformation. Current hypotheses are based on the assumption that metabolic alterations happen due to increased energy demands of cancer cells or decreased availability of nutrients or oxygen. Increased metabolism would be a mere consequence of cancer transformation. On the other hand, metabolism may be an active player in determining cell fate, such as malignancy progression. Indeed, it is true that metabolic changes confer a survival advantage when a tumor is already established [15].

It is generally thought that cancer cells make little use of mitochondria and mainly depend on anaerobic glycolysis for their energy demand even in presence of oxygen, a phenomenon called Warburg effect [46]. Nowadays, it is known that uncontrolled mitochondria bioenergetics has an important role in cancer metabolism and tumorigenesis [47], i.e., through the generation of ROS following disruption of regular mitochondrial homeostasis [48], strongly driving cancer progression [49].

Progressively several studies have been shedding light on the roles of mitochondria in the stemness state of cancer cells as well as on their contributions to metastasis and therapeutic resistance, along with strategies to identify and to eradicate these cells [50–52] by targeting their mitochondria [53,54].

It is known that $\Delta\psi$m is heterogeneous among cell populations and is related to the level of cell commitment and differentiation. Hippocampal neuroblasts, for instance, develop a significant $\Delta\psi$m increase when induced to differentiate in the presence of retinoic acid treatment. Accordingly, $\Delta\psi$m is also related to tumorigenic properties of stem cells. Among mouse embryonic stem cells, those with higher $\Delta\psi$m displayed augmented tumorigenic potential, although they presented similar levels of surface pluripotency marker expression and similar morphology [55].

Metabolic pathways and the distribution of mitochondria, which is related to the balance between symmetric and asymmetric divisions, tightly control stem cell populations [56]. Further, mitochondrial dynamics are involved in proliferating and quiescent states of cancer stem cells. A large fraction of mitochondria forms a tubular network in proliferating cells, while quiescent CSCs localized in the core of the tumorspheres, a highly hypoxic environment, are characterized by donut-shaped mitochondria [57]. In agreement, mitochondrial tubular morphology was donut-shaped under hypoxia-reoxygenation stress, a protective mitochondrial mechanism to aid adaptation and functional recovery [58].

In the context of metabolic reprogramming, CSCs have a mitochondria-centric energy metabolism, giving them the ability to consume limited available nutrients, such as fatty acids to generate ATP, NADPH, tricarboxylic acid (TCA) cycle intermediates, nu-

cleotide bases, electron acceptors and others, favoring cancer cell survival and proliferation signaling [49] and epigenetic regulation, but also by genetic-independent mechanisms tightly related to metabolic reprogramming [22,59].

In glioblastoma, glucose uptake and lactate output was even more expressive in CSCs than in the tumor bulk, in addition to upregulation of pyruvate dehydrogenase kinase-1 (PDK-1) expression levels [60]. However, lower oxygen and glucose consumption rates, intracellular ATP and ROS levels were observed in lung CSCs compared to differentiated cells, as well as oxidative phosphorylation preference for energy supply [61]. A chemoresistant stem-like side population (SP) within human tumors characterized by Hoechst33342 efflux capability presents higher glycolytic activity in comparison to their efflux-incapable counterparts [62]. Importantly, the proportion of SPs increases when glucose is abundant. This regulation depends on Akt pathway activation due to AMP-activated protein kinase (AMPK) suppression by increased intracellular ATP concentration [62].

Fatty acid (FA) metabolism includes anabolic and catabolic pathways essential to structure and sustain the cellular membrane, to supply energy and to produce intermediates mediating several signalling pathways. The fine-tuning balance between FA synthesis and oxidation can be easily perturbed by aberrant expression of the genes involved in these processes. Once this occurs, inadequate FA levels induce lipid accumulation and the general phenotypes of malignant cancers appear, being also strongly correlated with the presence of CSCs within the tumor cell population, as well as resistance to therapy [59].

The overexpression of lipogenic enzymes, such as fatty acid synthase (FASN) in several cancers has been correlated with cancer progression, poor prognosis and resistance to chemotherapy [63,64]. FASN is the enzyme whereby the condensation between acetyl-CoA and malonyl-CoA, at the final catalytic step of FA synthesis, produces palmitate, and has been indicated as an emerging target to cancer [65].

Glioblastoma stem cells expressing stem cell markers happen to be the same cells presenting upregulation of FASN. Interestingly, when the enzyme is inhibited by cerulenin, not only expression levels of stem cell markers, as well as the numbers of tumorspheres, GSCs proliferation and invasiveness are diminished [66].

4. P2X7 Receptor Relevance in Metabolism

The P2X7 receptor participates in regulation of mitochondrial functions, and the tone of ATP stimulation is crucial for determining downstream events. While tonic stimulation has a stabilizing effect on mitochondrial homeostasis, resulting in increased mitochondrial potential ($\Delta\psi$m) and oxidative phosphorylation efficiency; overstimulation has a killing effect, resulting in decreased $\Delta\psi$m, mitochondrial fragmentation and cell death [17,18]. Therefore, the expected effect of a tonic eATP (extracellular ATP) stimulation of P2X7 receptor in the tumor microenvironment is a combination of both increased glycolytic rate and oxidative phosphorylation efficiency, which contributes to ATP synthesis and anabolic responses [19].

Also, P2X7 receptor-induced effects on $\Delta\psi$m may play a role in determining stem cell fate, as well as tumorigenic potential. Indeed, $\Delta\psi$m is reduced in several cell types lacking P2X receptors, including HEK293 cells, human and mouse embryonal fibroblasts and microglia [17,18,67].

Although the role of P2X7 receptor in the balance between symmetric and asymmetric divisions is not yet clear, many pieces of evidence showed that P2X7 receptor stimulates the maintenance of cancer stem cells [14,67] and possibly asymmetric cell division [68]. An increase in ATP release and pericellular concentration was related to greater mitochondria numbers and activity in leukemia cells, resulting in cancer cell proliferation [69]. Importantly, P2X7 receptor-mediated effects on mitochondria morphology were reported following cell death or pseudoapoptosis induction [70]. However, respective functional implications need yet to be elucidated.

The downstream effects of P2X7 receptor activation culminate in metabolic changes, with some of them resembling the Warburg effect. P2X7 receptor expression in HEK-293

cells induced a metabolic reprogramming, which favored the adaptation of these cells to adverse conditions, such as growth in serum-starved [71] and low glucose media [72]. In addition to that, the presence of the receptor resulted in increased expression of glycolytic enzymes, while inhibiting pyruvate dehydrogenase and enhancing lactate production [72]. Such relevant findings where not only obtained with HEK-293 cells, but also similarly verified in a human neuroblastoma cell line [72].

P2X7 receptor expression was also associated with the rise of glycogen storage probably through down-regulation of glycogen synthase kinase 3β (GSK3β) activity [20].

P2X7 receptor stimulation in neuroblastoma cells increases intracellular ATP content [71] and enhances the PI3K/Akt pathway [20], suggesting a possible role in metabolic regulation of stem-like populations. In addition, glucose induces expression of glycolysis-related proteins, such as hexokinase-1 (HK-1) and pyruvate dehydrogenase kinase (PDK)-1 in CSCs, due to activation of the Akt pathway [62]. Interestingly, upregulation of a very similar set of proteins was verified upon P2X7 receptor stimulation in neuroblastoma cells, including PDK-1 [72], as shown in Figure 1.
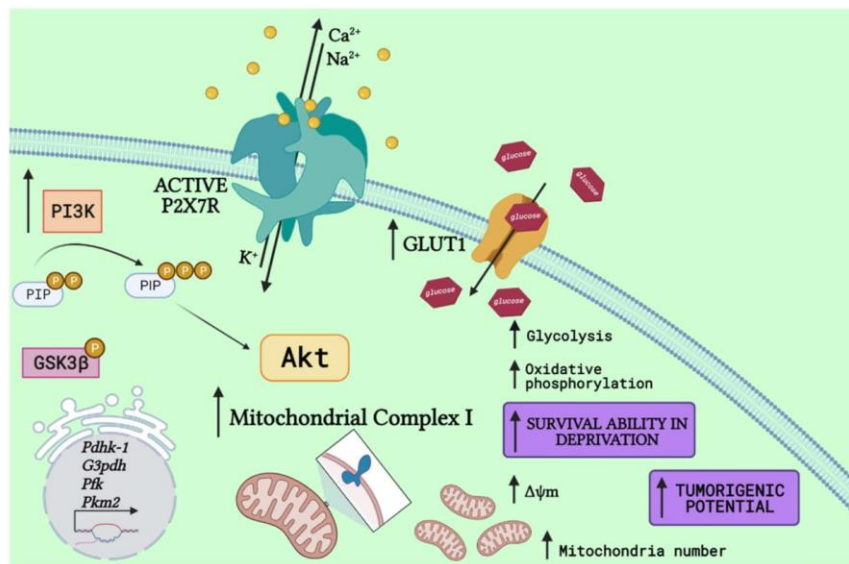
Figure 1. P2X7 receptor activation drives a metabolic shift. P2X7 receptor (P2X7R) activation drives PI3K activity, generating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and ultimately enhancing Akt signaling. Following, it drives upregulation of glycolytic-related enzymes, such as pyruvate dehydrogenase kinase-1 (PDHK-1) [72], a similar response to that observed in stem cells in the presence of glucose [62], and others, as glyceraldehyde 3-phosphate dehydrogenase (G3PDH), phosphofructokinase (PFK), and pyruvate kinase M2 (PKM2) [72]. Despite glycolytic improvements, the P2X7R is also related to increased expression of glucose transporters (GLUT) [72,73], increasing glucose uptake and oxidative phosphorylation, matched to higher expression of mitochondrial complex 1 and potential (Δψm), as well as mitochondria number [18] and increased glycogen storages through GSK3β phosphorylation [20]. Altogether, these metabolic shifts make cells highly capable of surviving in deprivation conditions and enhance the tumorigenic potential of cancer cells (Created with BioRender.com).

The P2X7 receptor has been related to FA metabolism in a way that P2X7KO mice presented reduction at mRNA and protein expression level of key enzymes, such as FASN and acetyl-CoA carboxylase (ACC). Besides, the absence of P2X7 receptor did not only

result in higher serum triglyceride and cholesterol levels, but also in glucose intolerance and insulin resistance [21].

Metabolic in vivo correlation with P2X7 receptor was observed, as body weight gain, abnormal lipid accumulation, adipocyte hyperplasia, increased fat mass and ectopic distribution was caused by P2X7 receptor loss of function in P2X7KO mice. Dysregulated energy homeostasis favored fatty acid oxidation not only in P2X7KO mice but also in WT mice treated with the selective P2X7 receptor antagonist A804598 [74], as seen in Figure 2.
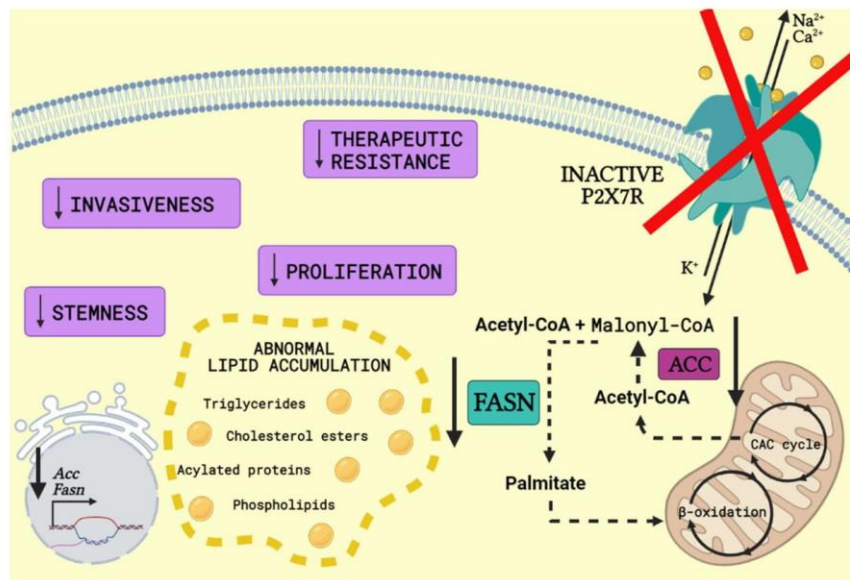
Figure 2. P2X7 receptor knockout or antagonism in FA metabolism. P2X7 receptor (P2X7R) knockout or antagonism reduces mRNA and protein expression level of fatty acid metabolism key enzymes such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN). ACC converts acetyl-CoA generated from citric acid cycle (CAC cycle) into malonyl-CoA. Following, FASN catalyses the conversion of acetyl-CoA and malonyl-CoA into palmitate, which enters mitochondria and goes through β-oxidation, producing long-chain saturated fatty acids. The knockout or antagonism of P2X7R surprisingly results in abnormal lipid accumulation, such as higher serum triglyceride and cholesterol levels, which is associated with hepatic steatosis [75]. Moreover, FASN inhibition is related to a decrease in the number of cancer stem cells, proliferation, invasiveness and resistance to therapy (Created with BioRender.com).

The same group stimulated P2X7 receptor in vivo with its agonist BzATP, which increased metabolic rate and O_2 consumption and decreased respiratory rate and upregulated NADPH oxidase 2 in gastrocnemius and tibialis anterior muscles [76]. These observations correlate P2X7 receptor activity with fatty acid catabolic pathway upregulation and cancer cell plasticity.

Malignant hematopoietic stem cells have also been shown to overexpress P2X7 receptors, if compared to their non-pathological counterpart [77,78]. In particular, acute myeloid leukemia (AML)-associated leukemic initiating cells (LICs) formed, proliferated, renewed and homed at the endosteal niche in a P2X7 receptor dependent fashion [79]. Interestingly, P2X7 receptor upregulated LIC growth and self-renewal via increased activity of phosphoglycerate dehydrogenase (PHGDH), a central enzyme in glycine and serine metabolism. Other key proteins of signaling pathways, which were in their expression

levels upregulated in AML by P2X7 receptors, include cAMP response element-binding protein (CREB) [79], Pre-B cell leukemia transcription factor 3 (Pbx3) [80] and c-myc [81].

5. Anti-P2X7 Receptor Drugs in Effectivity Studies or in Use for Cancer Therapy

The P2X7 receptor has become a target for anti-tumor therapy with promising outcomes in various tumors models [7]. Antagonists have been synthesized and described for in vitro and in vivo use to revert P2X7 receptor-mediated effects on the progression of cancer and non-cancer diseases. ATP analogues, such as periodate-oxidized ATP (oATP), have been used for P2X7 receptor antagonism in vivo [82]. However, this compound is not specific, as it also inhibits other P2 receptor subtypes. Brilliant Blue-G (BBG), a non-competitive antagonist of the P2X7 receptor, showed its biological activity by reverting loss of dopaminergic neurons in an animal model of Parkinson's disease [83] and blocking P2X7 receptor-promoted tumor engraftment and metastasis [6]. Although the BBG is approved for human use as food dye, this molecule is also not selective for P2X7 receptors, also inhibiting P2X4 and P2X1 receptors, besides affecting other proteins, including sodium channels [84] and pannexin-1 [85]. Another important factor is the limited systemic therapeutic use of BBG due to its blue staining properties of the retina. Formulations that improve its solubility and bioavailability might make BBG a more attractive molecule for in vivo applications.

Some P2X7 receptor antagonists, such as the allosteric-acting compounds A-740003 and AZ10606120, and the competitive-acting A-804598 [86,87] are more selective for the receptor, since they act in nanomolar concentrations. These antagonists were successfully used in in vitro and in vivo preclinical models to reduce P2X7 receptor-dependent cell growth and metastasis [7]. The oncological conditions, in which P2X7 receptor antagonism were efficacious in reducing cancer progression include, but are not limited to, breast cancer [88,89], melanoma [90–92], neuroblastoma [20], mesothelioma [8], glioma [93] and AML [79–81,92]. Notably, the P2X7 receptor antagonism reduced cancer aggressiveness acting at metabolic pathways in some of these models, such as AML, neuroblastoma and glioma [20,81,83,94].

The potent P2X7 receptor antagonist AZ10606120 significantly reduced proliferation of both U251 and surgically resected human high-grade glioma tumor cells [95]. Moreover, a high-affinity monoclonal antibody (4B3A4 mAb) was designed for blocking the human P2X7 receptor, by binding to its extracellular domain. P2X7 receptor activity was effectively blocked by the 4B3A4 mAb, once Ca^{2+} entry and YO-PRO-1 uptake stimulated by ATP were significantly reduced [94], making this compound promising for cancer therapy.

The CNS-penetrating drugs JNJ-42253432 and JNJ-47965567 were developed for treatment of brain diseases, including epilepsy [96]. Furthermore, P2X7 receptor inhibition has been subject in clinical trials for therapy of a number of inflammation-related disorders, including rheumatoid arthritis and inflammatory bowel disease [97]. Notably, the selective oral-administered AZD9056 P2X7 receptor inhibitor was effective in reducing inflammation in patients suffering from Crohn's disease in clinical trials [98]. Hopefully, P2X7 receptor antagonists currently tested for clinical safety, i.e., CE-224535 and emodin, will be evaluated in clinical settings for their capabilities in reverting tumor progression.

Non-functional variants of P2X7 receptor (nfP2X7R), which contain variations in the extracellular loop region, are broadly expressed in patient tumor samples and have been proven to be fundamental to cancer cell survival [99]. Antibodies developed to recognize nfP2X7R were called E200, for targeting the amino acid sequence in the range 200–216 [99]. These antibodies are being used as biological drugs for basal cell carcinoma treatment and have already passed phase I clinical trials, shown to be safe and tolerable [100]. Small-molecule antagonists, on the other hand, have resulted in several patents claimed by pharmaceutical companies, but they have not yet reached clinical trials [101]. However, animal model results have been promising [7,102]. An interesting review has gathered important studies and trials for drug development against P2X7 receptor in several diseases [103].

A recent study showed the significant anti-tumor activity of ATP-decorated and doxorubicin-loaded mesoporous silica with bio-mineralization of calcium carbonate against the doxorubicin-resistant highly aggressive and metastatic Dalton's murine lymphoma. The nanocomposite improved its capability of inducing apoptosis via P2X7 receptor activation, when compared to doxorubicin alone [104]. Doxorubicin and the similar chemotherapeutic daunorubicin are part of the anthracyclines drugs family, which increase extracellular ATP levels in the tumor microenvironment [105,106]. Interestingly, both doxorubicin and daunorubicin cellular uptake and consequent cytotoxicity are facilitated by P2X7 receptor variant A-promoted macropore opening [81,107]. On the other hand, the P2X7 receptor variant B protects cells from daunorubicin toxicity and even stimulates their proliferation, probably due to a daunorubicin-dependent ATP concentration increase in the tumor microenvironment. Consequently, in AML, chemotherapy with daunorubicin upregulated P2X7B receptor and downregulated P2X7A receptor expression, resulting in the overexpression of P2X7B receptors in AML-relapsing patients. In view of relapse and chemoresistance being the leading causes of death by AML, the P2X7B receptor is undoubtedly an attractive therapeutic candidate for this pathology [81].

While various studies in course, such as antibody development and clinical trials targeting the P2X7 receptor for cancer treatment, are promising, almost none of them addresses its isoforms in this big picture involving metabostemness, tumorigenesis and metastasis. However, for effectiveness of drug development, the whole scenario needs to be considered - not only the structure of the target of interest.

6. Final Remarks

Cancer is a complex and integrative disease hijacking entire body energy and functioning in order to grow and win. Therefore, interactions between the P2X7 receptor and its isoforms with metabolic regulation mechanisms are needed for successful interruption of the oncogenic process.

Aspects of P2X7 receptor structure and activity modulation by ligands within the extracellular milieu result from fatty acid metabolism. The P2X7 receptor C-terminal domain is implicated in most downstream effects of the receptor, including pore-formation and signal transduction [34], while the N-terminal domain may undergo alternative splicing dictating the sensitivity of different immune cells to extracellular NAD^+ and ATP [108]. Receptor modulation by membrane phosphoinositides (PIPn), anionic signaling phospholipids, happens through indirect interactions with the C-terminal tail [36].

While cholesterol-rich membranes may inhibit P2X7 receptor functions, the presence of lipids, such as sphingomyelin, phosphatidylglycerol, and phosphoinositides (PIPn), specifically $PI(4,5)P_2$, may promote its activity. Interestingly, PIPn modulation cross-talks with ubiquitous signaling pathways, such as those initiated by $PI(4,5)P_2$ hydrolysis. $PI(4,5)P_2$ is hydrolyzed by phospholipase C (PLC), generating inositol-triphosphate (IP_3) and diacylglycerol (DAG) [36,109].

In addition, the rate of agonist-evoked pore formation by P2X7 receptors can also be regulated by cholesterol-membrane levels. Acute depletion of cholesterol increases the rate of large pore formation, while its presence diminishes it, protecting cells from death [110].

Cellular membrane lipid composition is an important factor for pore formation. A truncated (lacking C-terminal) panda P2X7 receptor variant in controlled lipid composition exosomes stimulated with ATP alone was sufficient to allow a dye-permeable pore opening [109]. In addition, inhibition of the P2X7 receptor was induced by cholesterol interactions with TM2, preventing the receptor-induced large pore formation. Palmitoylation of cysteine residues in the C-terminal domain also prevents this interaction, protecting TM2 and allowing pore opening. In cholesterol-rich membranes, when the C-terminal is absent, TM2 remains unprotected and large pore opening is inhibited [109]. Excellent reviews are available to further delve into P2X and P2Y receptor pharmacology [111,112].

Metabolic targets associated with P2X7 receptor modulation, as well as its related cellular events are summarized in Table 1

Table 1. Summary of metabolic targets modulated by P2X7 receptor expression and/or activation and observed cellular responses.

Metabolic Target	P2X7 Receptor Effect	Related Cellular Events	Available Evidence
Complex I protein	Protein levels: Upregulated on P2X7R-expressing cells [18]	Increased mitochondrial potential, increased respiration	HEK 293 cells overexpressing P2X7R vs. wild-type; N13 microglia cells sufficient vs. deficient for P2X7R
Complex II protein	Protein levels: Downregulated on P2X7R-expressing cells [18]	Not reported	HEK 293 cells overexpressing P2X7R vs. wild-type
GLUT1	Protein levels: Upregulated on P2X7R-expressing cells [72]	Growth in absence of serum or low glucose; increased cellular ATP content	HEK 293 cells overexpressing P2X7R vs. mock-transfected cells; human neuroblastoma cells
GLUT2	Protein levels on cell surface: Downregulated by P2X7R activation [73]	Reduced glucose transport	Pharmacologically activated (BzATP 100 μM) intestinal epithelial cells (IEC)-6 and Caco-2 cells
G3PDH	mRNA expression levels: Upregulated on P2X7R-expressing cells [72]	Growth in absence of serum or low glucose; increased cellular ATP content	HEK 293 cells overexpressing P2X7R vs. mock-transfected cells; human neuroblastoma cells
PFK	Protein levels: Upregulated on P2X7R-expressing cells [72]	Growth in absence of serum or low glucose; increased cellular ATP content	HEK 293 cells overexpressing P2X7R vs. mock-transfected cells, in low glucose conditions; human neuroblastoma cells
PKM2	Protein levels: Upregulated on P2X7R-expressing cells [72]	Growth in absence of serum or low glucose; increased cellular ATP content; increased glycolysis	HEK 293 cells overexpressing P2X7R vs. mock-transfected cells, in low glucose conditions; human neuroblastoma cells
PDHK1	Protein levels: Upregulated on P2X7R-expressing cells [72]	Growth in absence of serum or low glucose; increased cellular ATP content; increased glycolysis	HEK 293 cells overexpressing P2X7R vs. mock-transfected cells, in low glucose conditions; human neuroblastoma cells
PDH	Enzyme activity: Downregulated on P2X7R-expressing cells [72]	Growth in absence of serum or low glucose; increased cellular ATP content; increased glycolysis	HEK 293 cells overexpressing P2X7R vs. mock-transfected cells; human neuroblastoma cells
GSK3β	Phosphorylated protein levels: Upregulated (reduced enzyme activity) on P2X7R-expressing cells/upon P2X7R activation [20]	Increased glycogen stores; tumor cell survival	Human neuroblastoma cells silenced for P2X7 by shRNAs vs. scrambled control; neuroblastoma cells pharmacologically modulated by agonists (BzATP or ATP) or inhibited by AZ10606120 or A740003
NADPH oxidase 2	Protein levels on skeletal muscle: Upregulated upon P2X7R activation [76]	Increased metabolic rate, O$_2$ consumption, decreased respiratory rate	P2X7 receptor systemic activation in mice (BzATP, 1 mg/kg)
ACC (acetyl-CoA carboxylase)	mRNA and protein levels: Downregulated in knockout mice [21]	Glucose intolerance, increased serum triglycerides and cholesterol levels	P2X7$^{-/-}$ mice vs. wild-type
FASN (fatty acid synthase)	mRNA and protein levels: Downregulated in knockout mice [21]	Glucose intolerance, increased serum triglycerides and cholesterol levels	P2X7$^{-/-}$ mice vs. wild-type
PHGDH	mRNA and protein levels: Downregulated in knockdown cells [79]	Lower serine levels; reduced migration, homing and self-renewal abilities	Leukemia initiating cells of mice with MLL-AF9 induced AML (Acute myeloid leukemia); P2X7 knockdown by shRNAs

The current hypothesis based on the assumption that metabolic adjustments occur due to increased energy demands of cancer cells or decreased availability of nutrients or oxygen put the metabolism in a passive position. These studies often do not consider that changes in metabolism may determine cell fate and stemness. Drug resistance must be faced not only as a product of natural selection, but most importantly as a phenomenon linked to cell plasticity that is triggered and reversed by environmental cues driving epigenetic changes. Uncovering how the environment may make cells more sensitive to chemotherapy is a current struggle that must be overcome. Cellular metabolism actively drives tumorigenesis and stemness, not only because of tumor establishment and cancer cells requirements. Recent studies try to understand how the P2X7 receptor modulates the metabolic reprogramming of cancer cells, such as intracellular ATP production, enabling cell division and cytoskeleton changes, which are necessary for tumor progression and metastasis. In addition to that, understanding how the receptor modulates metabostemness of cancer cells is also important. We already know that P2X7 receptor expression favors stemness of embryonic cells [44]. For that reason, together with the knowledge that the two functional P2X7 receptor isoforms may have opposite contributions to cancer biology, we believe the receptor is strongly related to the stemness balance within tumor mass, tumorigenesis, chemoresistance and metastasis [14].

The P2X7 receptor is an important molecule not only in oncogenic processes but also in normal tissues. Several P2X7 receptor antagonists have been synthesized for in vitro and in vivo use to revert P2X7 receptor-induced effects on the progression of cancer and other diseases and some of them underwent clinical trials. However, none of them has connected P2X7 receptor expression and activity modulation with metabostemness and cancer.

Metabolic reprogramming is essential for stemness, cancer metastasis and therapy resistance. Therefore, a solid understanding of P2X7 receptor expression, its regulation and their role in metabolic diseases and cancer metabostemness, as well as metabolic reprogramming are essential to ameliorate current anti-tumoral therapies.

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4.2.9. Complex diseases demand novel treatment strategies: understanding drug combination. *Drug Combination Therapy*, 2022.

Complex diseases demand novel treatment strategies: understanding drug combination

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Abstract
For many multigene or multifactorial diseases, the one-drug therapy for inhibiting a defined molecular target is often less effective than combined treatments. Typically, drug combination therapies are multitargeted, so the mechanisms or even interactions are often complementary. These drug-drug interactions may promote alteration of pharmacokinetic or pharmacodynamic activities of one drug by another drug. Other interactions may change the expected effect of medications through polymorphisms that alter the expression or activity of the drug-mediated enzyme and the cell signaling cascade, such as drug-gene interactions and drug-drug-gene interactions. The number of possible existing interactions requires appropriate methods of study. In this review, we summarized combination therapies for cancer, as well as for viral, cardiovascular, and neurological diseases. Here, we also highlight known methodologies, such as *in vitro* methods based on Loewe's and Bliss's pioneer models and *in silico* methods based on online available data. With more elaborate methods and reliable results, multitarget therapies through drug combinations may increasingly benefit patients suffering from complex diseases.

Keywords: drug combinations; multitarget therapy; combination therapy; drug-drug interaction; *in vitro, in silico*, methods and models

Introduction

Throughout history drug development research has been focused on identifying new agents directed against individual molecular targets, in an attempt to avoid any unspecific effects caused by mistargeting to other biological structures [1, 2]. However, biological organisms have many systems operating interconnectedly to protect the organism from malfunctioning [3]. Therefore one-drug to inhibit a defined molecular target is often less effective than combined treatments [3, 4]. Moreover, the complexity of some pathologies has challenged this paradigm, and the study of molecules hitting more than one target is now rising [5].

Multiple targeting strategies are used in clinical studies through a combination of various drugs that act distinctly on different targets of a sickness [6]. Multitarget therapies are repeatedly considered more efficacious, less toxic, and less vulnerable to resistance development, as a lower concentration of drugs is administered to the patient to reach similar or greater effect [2, 4]. This strategy is now standard in cancer, hypertension and viral infections, and is also of great promise for diseases such as Alzheimer's and tuberculosis [7–16].

Combination treatments are often multitargeted, but the two denominations are not synonyms. While a multitargeted agent may be a unique molecule with a suitable combination of activities, combination therapies combine two or more drugs that can act through the same molecular targets, and have complementary mechanisms or even unknown interactions [4].

Combining drugs raises many challenges, as each drug has different properties, such as bioavailability and metabolism, which leads to an exponential interest in the development of medicines based on a single molecule that acts on more than one specific target of disease [6]. The search for combined drugs targeting complementary mechanisms requires a large-scale search of a vast world of possible target combinations, but can also reveal potential synergies or unexpected interactions between disease-relevant pathways, leading to a greater understanding of disease biology [4].

According to Zimmerman et al., there are three categories of multitarget drugs that can be described by their relationship with the target [4]. In the first type, the therapeutic effect is given by separate targets that trigger individual signaling pathways in the cell; in the second type, the effect on one target favors the action on a specific second target; and in the third type, the coordinated action on a single target or a cell complex produces the therapeutic effect.

Therefore, when the interaction between two drugs reaches an effect larger than the sum of the independent effects, it is synergistic; if equal, it is addictive, and if it is smaller than the sum, it is antagonistic. However, defining the sum of two independent effects is actually more challenging than it seems: it is not feasible to simply consider the algebraic sum, for instance, when cytotoxic effects are expressed as fractions or percentages. The definition of additivity is then crucial for establishing mathematical and empirical models to study drug-drug interactions. Despite the existing mathematical approaches to predict interactions between drugs, the increase in drug combination treatments for known diseases has spurred the development of new computational methods to foresee efficient combinations [17].

Currently used drug combination therapies

Cancer

Most cancer treatment strategies include methods such as surgery and radiotherapy-for locoregional tumors-and systemic therapies for advanced and aggressive tumors that include chemotherapy, targeted therapies, endocrine therapy, and/or adjuvant therapy. Most clinically relevant chemotherapy drugs used to fight lung, breast, ovarian, and pancreatic cancers primarily target DNA molecules, such as platinum-based drugs (i.e., cisplatin, oxaliplatin, and carboplatin). These drugs cause a plethora of DNA lesions that, if not promptly repaired, induce tumor cell death. In other words, the main cytotoxicity mechanism of antitumor agents occurs through DNA damage.

Although some malignant tumors respond well to only one treatment strategy, other tumors require combined treatment with synergistic or additive effects for antitumor efficacy, since cytotoxic effects to healthy body tissues can overcome the effectiveness limited by primary and acquired drug resistance [18].

Furthermore, resistance to chemotherapy is estimated to be the cause of therapeutic failure in 90% of patients with metastatic cancer [19]. These limitations are based on the traditional use of chemotherapy to control the disease in a systematic way; therefore, radical and/or strategic changes in the treatment protocol for these tumors are necessary, to achieve more effective clinical results.

Certainly, if we could overcome drug resistance, the impact on patient survival would be immense. An interesting alternative often used in the clinic is the combination of different chemotherapies to enhance the therapeutic effect of these drugs. The modalities of drug combinations are based on their complementary mechanisms of action, to create an appropriate therapeutic program considering the characteristics of the patient and tumor. What is sought with this therapeutic strategy is that the cytotoxic effect of the drugs together is synergistic. Thus, it is possible to administer lower doses and obtain the same or better therapeutic effects and, at the same time, reduce side effects often associated with chemotherapy.

Neoadjuvant treatment for the most aggressive breast cancer subtype, triple-negative breast cancer, is an example of a current regimen that consists of drug combinations of anthracyclines plus cyclophosphamide followed by platinum compounds and taxanes which significantly increase pathologic complete response and reduce the risk of recurrence [20].

Anticancer therapeutic regimens must also consider the impact of systemic therapies on the tumor microenvironment, which is often established by the tumor-host relationship. The components of the microenvironment have great importance in tumor growth and progression. Endothelial cells, stromal fibroblasts, specific natural killer cells, tumor-associated macrophages, and specific lymphocytes stand out in the tumor microenvironment. Currently, the understanding of the tumor-host interaction has been used as a determinant of clinical evolution and types of responses to treatment for human neoplasms [19–22].

Some drugs classified as antiangiogenic, such as sunitinib, sorafenib, and bevacizumab, are used in combination to manipulate the tumor-host interaction favoring the reduction of the tumor, through the biological targeting of the endothelial cell and interruption of the vascularization associated with the tumor [23].

Furthermore, new methods of therapy based on activation of the immune system have added many benefits to patients with several types of cancer in relation to the duration of response and survival time, especially when combined with chemotherapeutic drugs.

Nevertheless, chemotherapy is not restricted to being cytotoxic to cancer cells; it also affects other cells, such as endothelial cells and immune cells in general [24]. The same happens with antiangiogenic and tumor-specific monoclonal therapies [25, 26]. Any changes in the cancer biology process will affect other elements present in the tumor microenvironment and host elements.

Thus, a careful assessment of the impact of combinations between drugs already established, including chemotherapy, and new drugs designed to modulate the immune system, such as vaccines, checkpoint inhibitors, and adoptive cellular therapy, has been necessary to generate a more effective multimodal therapeutic integration in the treatment of cancer.

Moreover, genetic variability in combination or not with other factors, such as medical conditions or even environmental influences, can alter the response of an individual to treatment [27, 28]. Thus, each patient in a large population can respond differently to the same medicine.

Pharmacogenomics studies the variability in response to drugs due to genetic variations that are often related to single-nucleotide polymorphisms (SNPs) causing mutations in genes, for example,

multidrug resistance (MDR) 1, multidrug resistance protein (MRP) 1, and MRP2, which are associated with resistance to drugs or other genes involved in drug metabolism and transport [29, 30]. Such mutations can change the gene expression or structure of proteins involved in pharmacokinetic and pharmacodynamic parameters, which explains why a treatment never has the same effect in several patients and why different patients can present distinct adverse drug events [28].

Adverse drug events may significantly change the expected effect of prescribed medications and are often related to so-called drug-gene interactions (DGIs) and/or drug-drug-gene interactions (DDGIs) [30, 31]. The genome affects metabolism through polymorphisms that alter a drug's metabolic enzyme by decreasing or increasing its function. If a SNP causes the expression of a low function enzyme, for example, this enzyme may reduce the metabolism of the administered drug through a DGI (Figure 1A) [30]. Then, this low-function drug's metabolic enzyme can interact with another drug by DDGIs by inhibitory or inductive interactions, or also an interaction of phenoconversion [30]. The interaction of the enzyme with an enzyme-inhibitor drug or an enzyme-inducing drug may promote reductions or increases in drug metabolism, respectively, leading to pharmacokinetic and pharmacodynamic changes in the medication (Figure 1B) [30].

Considering that DDIs and DDGIs are linked with adverse drug responses in the treatment of several diseases, the identification of clinically relevant interactions becomes increasingly important. Currently, pharmacogenomics enables the investigation of genes and genetic variants and the identification of particular genetic loci of an individual and/or related to a disease that determines the drug response of patients [28]. Thus, information such as this will allow individual development and planning of more effective and accurate therapeutic approaches for each patient shortly.

Cancer vaccine and chemotherapy combination Currently, the potential use of therapeutic combinations that incorporate chemo and radiotherapy has been discussed with a view to synergy with antitumor vaccines. In general, chemotherapy is considered immunosuppressive because it acts on dividing cells, including bone marrow cells and peripheral lymphoid tissue. The use of cancer vaccines as an immunotherapeutic strategy is based on their ability to capture and present tumor proteins, triggering a strong and effective immunogenic response, through the activation and expansion of effector cells such as Th1 lymphocytes, cytotoxic T lymphocytes (CTLs), and natural killer cells. The recognition of peptide complexes of tumor cells via major histocompatibility complex class-I (MHC-I), through the T-cell receptor, promotes CTL activation and releases cytotoxins (perforin and granzyme), destroying malignant cells. However, this treatment alone is insufficient to generate tumor regression, especially in advanced diseases [32, 33].

Several studies have shown benefits in the combination of vaccines and some chemotherapy drugs in low doses against some tumors, including breast lymphomas [34]. The first Food and Drug Administration (FDA) approved antitumor dendritic cells (DCs) is a first-generation vaccine, called Sipuleucel-T (Provenge®), which was approved for all types of cancer. In a randomized phase III study, patients with prostate cancer hormone-resistant or metastatic castrating were treated with DCs autologous cells obtained from peripheral blood cells cultured in vitro with granulocyte-monocyte colony-stimulating factor (GMCSF) and a prostate-specific antigen, acid phosphatase (PA2024). The cell immunotherapy induced an immune response in these patients, reducing the risk of death by 22% and increasing overall survival (OS) in 4.1 months compared with the placebo group. Despite the substantial difference in OS, the single use of Sipuleucel-T does not alter disease progression in the short term. A phase III trial showed more benefit in survival when patients received docetaxel after vaccine treatment [32, 33].

T-regulatory cell modulation and chemotherapy combination Several clinical studies have demonstrated an important synergistic effect between chemotherapy and checkpoint inhibitors when used in combination. By causing the death of tumor cells, chemotherapy promotes the release of antigens that are presented by antigen-presenting cells, making the tumors more immunogenic for the performance of immunotherapeutic agents. This combination has been reflected in the increased response rate and progression-free survival of several types of tumors [25, 32, 33, 35–38]. With technological development and greater knowledge of the immune system, inhibitory pathways which attenuate this immune response was discovered. Drugs acting on this interface of the immune system are called immunological checkpoint inhibitors [34].

The cytotoxic T lymphocyte antigen 4 (CTLA-4) molecule expressed in T lymphocytes after being activated, has great homology with the CD28 molecule. It also has a greater affinity for CD80/CD86 than the CD28 molecule, activating an immunoregulatory response that inactivates the T4 lymphocyte response [39–41]. Ipilimumab was the first monoclonal antibody against CTLA-4. Its use proved to be effective in the regression of melanoma, renal carcinoma, prostate cancer, urothelial carcinoma, and ovarian cancer [42, 43]. In addition, the combination of ipilimumab with other immune checkpoint inhibitors had positive anti-cancer effects for metastatic melanoma, metastatic colorectal cancer, and advanced renal cell carcinoma [44].

The programmed death 1 (PD-1) pathway is a checkpoint that limits mediated immune response by T cells. Its ligands, PD-L1 and PD-L2 on cancer cells bind to the PD-1 receptor on immune cells and induce PD-1 signaling for T cell "exhaustion", reversible inhibition of T cell activation and proliferation [43, 45]. Multiple antibodies (PD1 or PDL1-inhibitors) are in clinical phase studies [46, 47]. Pembrolizumab and Nivolumab are PD1-inhibitors approved for the treatment of advanced melanoma, renal cell carcinoma, and non-small cell lung cancer (NSCLC), as clinical studies have shown improvements in OS [48, 49]. However, many patients do not benefit from PD1 or PDL1-inhibitors. Thus, the combination of immunotherapy and chemotherapy are options for first-line treatment in patients with metastatic NSCLC without epidermal growth factor receptor (EGFR) mutation or anaplastic lymphoma kinase (ALK) translocation which has improved OS, progression-free survival (PFS), and overall response rate [44, 50].

Although immunotherapy has benefited cancer patients, it is worth mentioning the high cost of this therapy, which can limit access to patients [51].

Targeted therapy plus immunotherapy Targeted therapies have been used in several types of tumors with very expressive clinical responses rates. Targeted therapy uses small molecules or monoclonal antibodies to attack features unique to cancer cells as growth, division, and spread. Nevertheless, the response rate is often transient, causing the escape by tumor cells through the generation of resistance mutations, months after starting treatment. By contrast, immunotherapies have shown more durable responses in several types of tumors, mainly in combination with other available therapies. Several clinical studies corroborate this new approach-mainly through a deeper understanding of the mechanisms of tumor immunity and their interaction with genomic mutations, generating an important impact on therapy directed to the tumor microenvironment. To date, translational studies have shown clear benefits in combining target therapies with checkpoint inhibitors (anti-PD1 and PDL-1) [52].

The results of preclinical and translational studies revealed interesting effects of combining mitogen-activated protein kinase (MAPK) inhibition, as BRAF ± MEK inhibitors with checkpoint inhibitors (anti-CTLA-4 and/or anti-PD-1/PD-L1). Such combinations had a significant effect on the immunomodulation of melanoma patients increasing the proportion of CD8+, CD4+, and PD-1+ T lymphocytes in the tumor microenvironment, consequently increasing the anti-tumor immune response [48].

Currently, clinical trials combining anti-vascular endothelial growth factor (VEGF) and anti-PD-1 therapies have shown good clinical benefits compared to monotherapy, in advanced NSCLC and melanoma patients [37, 53]. The therapeutic atezolizumab (anti-PD-L1) and bevacizumab (anti-VEGF) combination can increase the immune system's potential by the ability of atezolizumab to activate T cell responses against the tumor [53]. Another study

evaluated the combination of ipilimumab and bevacizumab versus monotherapy with ipilimumab alone in advanced melanoma. The combination therapy increased the expression of adhesion molecules and intratumoral CD8$^+$ T-lymphocytes, improving the clinical responses [54].

In renal cancer cells, the combination of nivolumab (check-point inhibitor; anti-PD1 antibody) and ipilimumab (check-point inhibitor; anti-CTLA-4 antibody) was tested versus sunitinib in metastatic patients not previously treated (Checkmate 214 study). The combined treatment was able to reduce the risk of death by 37%, increasing the response rate from 27% to 42% and from 8.4 to 11.6 months PFS in the intermediate-risk and poor population. PD-L1 expression seems to be related to the best response to immunotherapy. Meanwhile, in a subgroup analysis, patients at favorable risk responded better to sunitinib, with a higher response rate and higher PFS [54]. Another phase III study, IMmotion 151, evaluated the combination of atezolizumab (anti-PDL1 antibody) and bevacizumab (anti-VEGF antibody) versus sunitinib at the first line, showing an increase in PFS manly in immunohistochemical positive PDL-1 ($\geq$ 1%) samples [55].

Viral diseases

In human viral diseases, such as acquired immunodeficiency syndrome (AIDS) and hepatitis, the progress of treatment has also relied on the use of combination therapies. The strategies for treatment of human immunodeficiency virus (HIV) infection, the agent behind the AIDS disease, changed with the knowledge that viral replication occurs during the years before the development of the clinical disease, with a progressive decline of the immune function by latent infection of CD4 T cells [56, 57]. Antiretroviral therapy (ART) also called HIV regimen, consists of treatment with the combination of two or three medicines. By 1998, drugs licensed in the US for use in combination therapy to HIV included nucleoside and non-nucleoside reverse transcriptase inhibitors, integrase inhibitors, and protease inhibitors [56, 58]. ART, outstandingly, improves the prognosis of individuals infected with HIV, being efficient by suppressing viral load, preventing viral resistance demonstrating low toxicity in a short time, and good tolerance by patients [58, 59]. After some years of studies, ART consisting of combined drugs, such as tenofovir alafenamide, ritonavir, zidovudine, dolutegravir, bictegravir among others, has resulted in a survival range for patients living with HIV similar to HIV-uninfected people, and reduction of the viral transmission [57, 58]. Other antiviral therapies of combined drugs have only been explored in recent years for conditions such as chronic hepatitis C and B [60].

A leading cause of severe liver disease, hepatitis C virus (HCV) infection can result in cirrhosis and hepatocellular carcinoma in patients [61]. After the discovery of interferon alfa as the principal agent in HCV therapy, the development of direct-acting antiviral agents (DAAs) has provided an important advance in the treatment [62]. Although interferon-based HCV therapies offer a 40% cure for the most difficult to treat genotype-1 infection, treatment with interferon-free DAA regimens, such as combinations of DAAs and medicines, has been successful in most patients [61]. Nevertheless, the implementation of DAAs is still a challenge due to the different six clinical genotypes of HCV. The first oral regimen of sofosbuvir and ribavirin that was approved by the US for genotypes 1–4 became the standard treatment for genotypes 2 and 3, but its efficacy was not sufficient in genotype 1 and did not have sufficient evidence to genotypes 5–6 [61]. In the case of hepatitis B virus (HBV) infection, various combination therapies have been evaluated over time but just a few of them have been shown to induce higher rates of response as compared to monotherapy [60]. Currently available therapeutic options include medications such as interferon or pegylated interferon, nucleotide, and nucleoside analogues but although when combined they achieved good results it is still needed more trials [63, 64]. Many agents available for the treatment of HBV are also part of combination regimens for HIV infection. Thus, the combination therapy option is especially attractive for HBV, since interferon monotherapy has therapeutic effectiveness limited to 25% to 40% [63].

Cardiovascular diseases

Hypertension is considered one of the strongest risk factors for different cardiovascular diseases because of the direct relationship between blood pressure and cardiovascular events [65]. Decades of research have offered antihypertensive treatment a lot of medications that can reduce elevated blood pressure by being used alone or in combination with one another [66]. Since the treatment demands a quick normalization of blood pressure rate, the achievement of the ideal blood pressure is difficult in most patients [67]. Recently, evidence indicates that the use of combined drugs at the beginning of treatment can normalize blood pressure more quickly, reducing significantly major adverse cardiac and cerebrovascular events [65]. The fact that hypertension is multifactorial, therapies based on drug combination may be favorable since the combination of the agents may lead to better blood pressure control by the action of a supposed complementary mechanism [67]. Nowadays, the combination of a blocker of the renin-angiotensin system, generally one of the angiotensin-converting enzyme (ACE) inhibitors of first-generation as captopril, enalaprilat, and lisinopril, with a calcium channel blocker of the class of dihydropyridine or non-dihydropyridine; or an ACE inhibitor with a diuretic for reducing sodium and water levels in the body are prescribed. These drug combinations have been successful to promote the best cardiovascular protection and good therapeutic results for providing greater antihypertensive effectiveness and fewer side effects than the use of high doses of monotherapy [65, 66].

Neurological diseases

Diverse neurological disorders are multifactorial, thus single-target drugs are mostly inadequate to achieve satisfactory therapeutic effect [68]. In addition, the therapeutic strategy available for some well-described neurodegenerative diseases is still based on the treatment of symptoms. In this topic, we will highlight some common drug interactions that are beneficial or harmful to affected patients by neurological diseases, such as depression and Alzheimer's disease.

Major depressive disorder, also commonly called depression, affects increasingly amount of the population year after year. The cause is still unclear; however, many drugs are available to fight the disorder. One of the most recent challenges on psychopharmacology is the drug-drug interactions (DDIs), since psychiatrists are treating patients with increasing complexity medication [69, 70]. DDIs are defined by the alteration of the pharmacokinetic or pharmacodynamic activities of one drug by another drug [71, 72]. In pharmacokinetic interactions, a drug can change the absorption, distribution, metabolism, and elimination (ADME) of other drugs reciprocally, besides affecting its concentrations at the sites of action (Figure 2). Knowledge of the mechanisms behind DDIs and their consequences is crucial for the development of combination therapies, since the most widely used treatments may have secondary pharmacologic characteristics that could induce or inhibit the activity of drug-metabolizing enzymes or transport proteins [73]. According to Greenblatt, metabolic inhibition results from an inhibitory DDI that may reversibly or irreversibly affect the drug-metabolizing enzymes themselves, frequently the cytochrome P450 enzyme (CYP450) (Figure 1) [73]. For instance, the administration of fluoxetine to a patient that already has a titrated dose for Nortriptyline will produce clinical tricyclic toxicity, because fluoxetine is an inhibitor of the enzyme that metabolizes Nortriptyline, which the consequence is higher levels of Nortriptyline [74]. In addition, the reverse situation will also induce toxicity, for example, the administration of Phenytoin (degradation enzyme-substrate) to fluoxetine or fluvoxamine-treated patient [75, 76].

Furthermore, some drugs can induce the degradation of another one, by activating its metabolic enzyme, as previously discussed [77–79]. Such a situation happens in the co-administration of Phenytoin and Risperidone [75]. The first is an inducer of CYP450 3A4 enzyme, the same that metabolize risperidone. Therefore, the patient will need

higher doses of risperidone to achieve the same effect. As well as Alprazolam addition to the carbamazepine-treated patient [80].

Another classic situation is the change of habit of a smoker. Smoking tobacco is a potent and ubiquitous inducer of CYP4501A2, which metabolizes some drugs such as Clozapine [81]. Once the patient decides to quit smoking, the blood levels of clozapine will rise causing morbidity. Accordingly, it is highly recommended for clinicians to prescribe medicaments within a given class with a low likelihood of producing DDIs, likewise citalopram/escitalopram among the selective serotonin reuptake inhibitors (SSRIs), mirtazapine or venlafaxine among the non-SSRI antidepressants, pravastatin among the statins, and azithromycin among the macrolides [82–84, 77].

Although current medications cannot cure Alzheimer's disease or stop it from progressing, they may help lessen symptoms, such as memory loss and confusion, for a limited time. Therefore, in order to treat Alzheimer's symptoms in early to moderate stages, there is a class of drugs called cholinesterase inhibitors.

Cholinesterase inhibitors are usually prescribed to treat symptoms related to memory, thinking, language, judgment, and other cognitive processes. Some examples of this category are donepezil, galantamine and, rivastigmine (see Miranda et al. for a complete review on Alzheimer's drug discovery) [85]. However, cholinesterase inhibitors are not effective enough for advanced stages of Alzheimer's disease, given that, the current FDA-approved treatment is the combination of donepezil with memantine [86].

Memantine regulates the activity of the neurotransmitter glutamate, by antagonizing one of its receptors, the NMDA receptor [68, 13, 86]. Though powerful, this treatment still displays notable disadvantages, including increased toxicity such as seizures, irregular heartbeat, dizziness, and stomach ulcer; and treatment costs [68, 86]. Opposing, a clinical trial led by Robert Howard in 2012 did not show any significant benefits of the combination of donepezil and memantine over donepezil alone [87]. Taken together, these data emphasize that the combination treatment for neurological diseases should be well studied.

Studying drug combinations

In vitro studies
Combining drugs, resolving their individual and combined effects, and translating the results into clinically plausible recommendations is indeed a challenge. In pharmacokinetic DDI studies, it is possible to measure the expression of enzymes, transporter systems, and human cellular fractions, and their specific interactions with drugs of interest [88]. On the other hand, pharmacodynamic interpretations of synergism and antagonism demand well-described theoretical approaches, usually based on a reference model, which establishes an index of additivity or null interaction [89, 90].

Null models define an ideal "non-interacting" system, from which definitions of additivity and synergism are derived. In 1992, scientists from all over the world reunited to reach a common understanding of the terminology employed in drug combinations research, leading to the Saariselkä agreement [91]. The consensus reached was that there was no one-best model and that the two approaches predominantly used in the research field are appropriate for different sets of applications. Next, we will briefly discuss these approaches: the Loewe additivity and the Bliss independence models [92, 93].

Loewe's model The null model proposed by Loewe is based on the sham principle, which postulates that one agent cannot interact with itself [92]. Thus, if two drugs do not interact, effects obtained by their combination would be equivalent to those observed if one drug is combined with itself. This is the principle of concentration addition, the reason why Loewe's additivity model is also known as the concentration addition method [92]. For determining a reference non-interactive scenario among two compounds, one should determine complete dose-effect or dose-response curves for both, which will correspond to the first line and column of a matrix of combinations [94].

Some criteria must be met so that these assumptions are accurate: the principle of dose equivalence, similar pharmacodynamic properties, and a constant potency ratio. In summary, dose-response curves must be parallel, as shown in Figure 3. This ensures that a concentration α of a compound A (A_α) able to disclose an effect y is equivalent to a concentration β of a compound B (B_β) able to disclose the same effect y. Let's assume that both concentrations individually result in the half-maximum effect (i.e. $y = 0.5$; α and β are the IC50 values of compounds A and B, respectively). If a concentration $a =$ is used in a co-exposure experiment and the principle of dose equivalence holds up, to produce the same half-maximum effect, the concentration of the compound B must be $b = B - xB$, so that the following mathematical correlation applies:

$$\frac{a}{A_\alpha} + \frac{b}{B_\beta} = 1$$

This equation is known as the median-effect equation. If this correlation applies, the combination is considered additive. Thenceforth, it is possible to define relationships between pairs of several dosages tested. In 1983, Chou and Talalay proposed an algorithm based on this equation called "combination index" (CI), which categorizes the combinations as synergic (CI < 1), antagonistic (CI > 1), or additive (C = 1) [95, 96]. Moreover, when several values of y are considered, the isobole equation arises:

$$\frac{a}{f_A^{-1}(y)} + \frac{b}{f_B^{-1}(y)} = 1$$

A graphical representation called isobologram plots growing concentrations of the compounds analyzed to draw a straight-line distinguishing additive non-interaction from synergistic and antagonistic interactions (Figure 3A) [97–99].

Derived from Loewe's model several others emerged, such as Tallarida's model and Hand's model [97, 100–102]. For constant potency ratio drugs, the Loewe, Hand, and Tallarida models are considered very similar [94]. Although, Hand suggests building dose-effect curves for the combination of full and partial agents in instantaneous time. This characteristic is essential in biochemical models where molecular reactions happen all the time even though do not give knowledge about them [94].

Bliss's model The Bliss model follows an effect-based null strategy, relying on the principle that drugs act independently through different sites of action and together assemble a combined response [93, 94, 99]. Based on a probabilistic assumption and considering the effect as a fraction of the maximal possible response, the additive effect of a drug A on a dose α (A_α) and a drug B on a dose β (B_β) would correspond to the observed effect of A_α plus the effect of B_β on the remaining fraction. For didactic purposes, consider that the measured effect is cell survival. If A_α impairs the viability of a fraction x of the observed cells while B_β reaches a fraction y; the combined effect in a non-interaction context would correspond to the sum of:

a. The cell viability reduction produced by A_α alone = x, and
b. The cell viability reduction produced by B_β in the remaining population of cells = $(1 - x) * y$

Thus, a general formula could be written as:

$$A_\alpha B_\beta {}_{BLISS} = A_\alpha + (1 - A_\alpha) x B_\beta$$
or
$$A_\alpha B_\beta {}_{BLISS} = (A_\alpha + B_\beta) - A_\alpha x B_\beta$$

If doses A_α and B_β act independently, the surviving fraction of the cells after simultaneous administration will be the same as cells that survived only drug A or drug B treatments. In other words, after the effect of drug A, the remaining fraction x of cells is then affected by drug B that produces the fraction y, acting independently. However, if there is synergy, the fraction of surviving cells after the simultaneous administration of the drugs will be smaller than cells that have been treated only with drug A or drug B [103]. If there is antagonism, the fraction of surviving cells will be bigger than cells treated only with drug A or drug B [103]. Thus, there are three basic types of drug combinations in the Bliss model [93]: independent, similar, and synergistic/antagonistic. If the combined drugs present different

modes of action and act independently, can be defined as independent; if the drugs present similar effects and interact independently, can be classified as similar; finally, if the effect is more or less potent than individual effect, the response is considered synergistic or antagonistic [3, 93].

Such Bliss's model suggests that two drugs reach their effects independently, the Highest Single Agent (HSA) model also suggests that the expected combination effect should be equal to the higher effect of the individual drugs [104]. Therefore, any additional effect over the higher single drug is considered synergy [105].
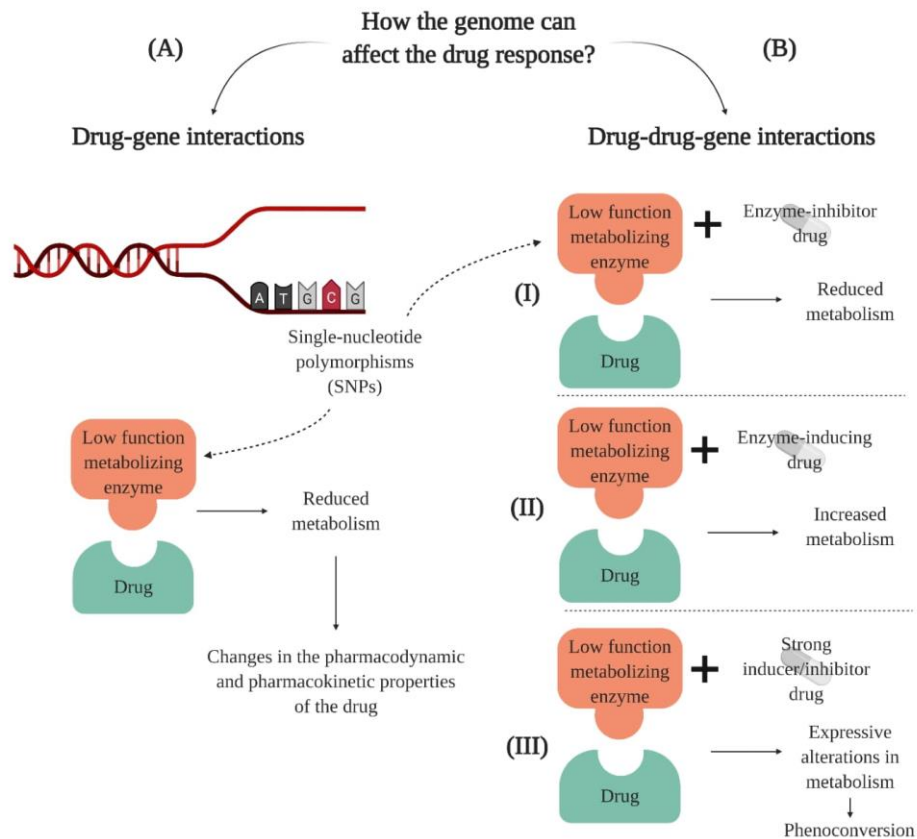

Figure 1 Representative scheme of drug-gene and drug-drug-gene interactions. (A) In drug-gene interactions, a single-nucleotide polymorphism (SNPs) in DNA can cause dysfunction of a drug-metabolizing enzyme, such as CYP450. For instance, a reduction in its activity can result in a reduction in the metabolism and also in the response of the drug once its pharmacokinetic and pharmacodynamic properties would be changed [28]. (B) In drug-drug-gene interactions, the effect of one drug can be affected by the interactions between the mutated drug-metabolizing enzyme and another drug, causing (I) inhibitory, (II) inductive, or (III) phenoconversion interactions [30]. Interactions of inhibitory and inductive character are caused by drugs that promote a reduction or increase of the metabolism of the drug, respectively, leading to changes in the pharmacokinetics and pharmacodynamics properties [30]. The phenoconversion interaction is also inhibitory or inductive, but it is usually related to strong drugs that can promote expressive changes in metabolism resulting in a change of the phenotype [30]. Basically, drug-gene interactions occur when the genome affects the ability of a patient to metabolize a drug, while drug-drug-gene interactions occur when the genome and another drug together affect the metabolism of a medication [123]. Image created with BioRender.
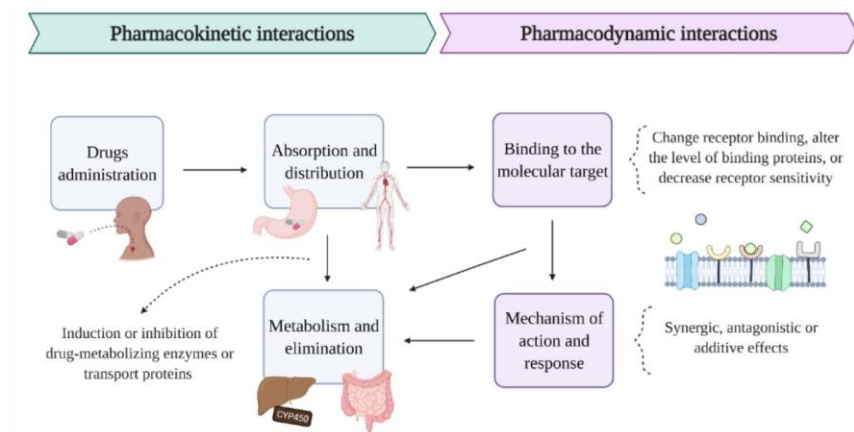
Figure 2 Pharmacokinetic and pharmacodynamic drug interactions. In pharmacokinetic interactions, orally administered drugs may change the absorption in the gastrointestinal system and the consequent distribution into the bloodstream [71]. Even after being absorbed, the action of the drug may change before its elimination from the body, since the metabolism of drugs in the liver can pass through alterations if one of the drugs inhibits or induces transport proteins and/or drug-metabolizing enzymes, such as CYP450 [73]. In pharmacodynamic interactions, the effect of one drug can be affected by the administration of another drug through synergistic, additive, or antagonistic effects that can directly affect the binding of the drugs to their molecular receptors and also the levels or sensitivity of the receptor [72]. These interactions can cause an alteration in the cell signaling cascade responsible for the mechanism of action and response of the drug in the body. Image created with BioRender.

In silico methods

To identify new combinations, systematic high-throughput in vitro testing of pairwise drug combinations can be applied. But this can be time and resource-consuming as for every 100 drugs there are 4, 950 possible drug pairs combinations that could be tested; if we are searching for combinations of three drugs the number rises to 161, 700 possibilities. Thus, to decrease the number of combinations to be tested in vitro, computational approaches can be used to tackle this issue and prioritize more promising combinations, although the development of models to foresee drug combinations with high accuracy is not an easy task.

In order to improve the understanding of drug combination synergy and facilitate the development of novel and better computational tools, in 2015 a challenge called the DREAM Challenge was launched and brought relevant advances on the field [106]. For this challenge the AstraZeneca dataset was created, containing 11, 576 experiments from 910 combinations across 85 cancer cell lines, that were provided to the 160 participating teams to train new models. The best performing team applied a random forest algorithm, a Machine Learning (ML) approach, to perform the predictions and achieved a high performance compared to the theoretical limit, using as input monotherapy data, gene interaction networks, drug target information, and the molecular profile of the 85 cell lines to calculate the synergy scores [107, 108]. ML are algorithms that can derive models for classification, prediction, and pattern recognition from existing data; so, data such as the AstraZeneca set can be used to identify patterns on known synergic drugs to predict new and unknown synergic drugs based on these patterns [109].

ML algorithms have been widely applied to predict drug synergy for cancer treatment, as exemplified by the winning team of the DREAM challenge, especially due to the considerable amount of available data from this disease, which is essential and limiting to train new models [110–113, 107, 108]. Another example is DeepSynergy, which was the first method to apply Deep Learning to predict anti-cancer drug synergy, requiring the genomic profiles of cancer cell lines and compounds represented by their chemical descriptors to perform the predictions [114]. Further, to overcome the cancer data scarcity from some understudied tissues, such as bone, prostate, and pancreas, a deep neural networks model was developed to utilize information from the data-rich tissues (breast, kidney, skin, and lungs) and improve prediction accuracy [115].

Nevertheless, ML algorithms can also be applied to predict combinations for the treatment of other diseases, as shown by the computation model INDIGO-MTB that uses drug-gene associations inferred from transcriptomic data from Mycobacterium tuberculosis and experimentally measured drug-drug interactions to infer interactions between new combinations of drugs for the treatment of tuberculosis [116]. This model could also be applied to other pathogens with transcriptomic data available.

Network-based methods, which generally use information from genomic, chemical, and pharmacological properties to build a network representing the associations among drugs, proteins, and pathways, can also be very powerful tools [117]. One example is the method developed by Cheng et al. to identify clinically efficacious drug combinations that provided novel insights to understand drug synergy [118]. This method is based on the relationship between drug-target pathways and the disease pathways via network proximity in the human protein interactome built based on protein-protein interactions (PPI) experimentally confirmed. They found, using known drug combinations for hypertension and cancer, that to have synergism with lower adverse effects each drug must target different groups of interacting genes that are affected by the disease, and that drugs that target a similar group of genes have more adverse effects [118]. These results are corroborated by Qian et al., which also indicated that known adverse drug pairs tend to have more genetic interaction on their targets [119].

The method developed by Cheng et al. was later applied for drug repurposing for treatment of the novel coronavirus SARS-CoV-2, using virus-host interactome information from 4 human coronaviruses (SARS-CoV, MERS-CoV, HCoV-229E, and HCoV-NL63) and other viruses, and transcriptomics data in human cell lines [118, 120]. From
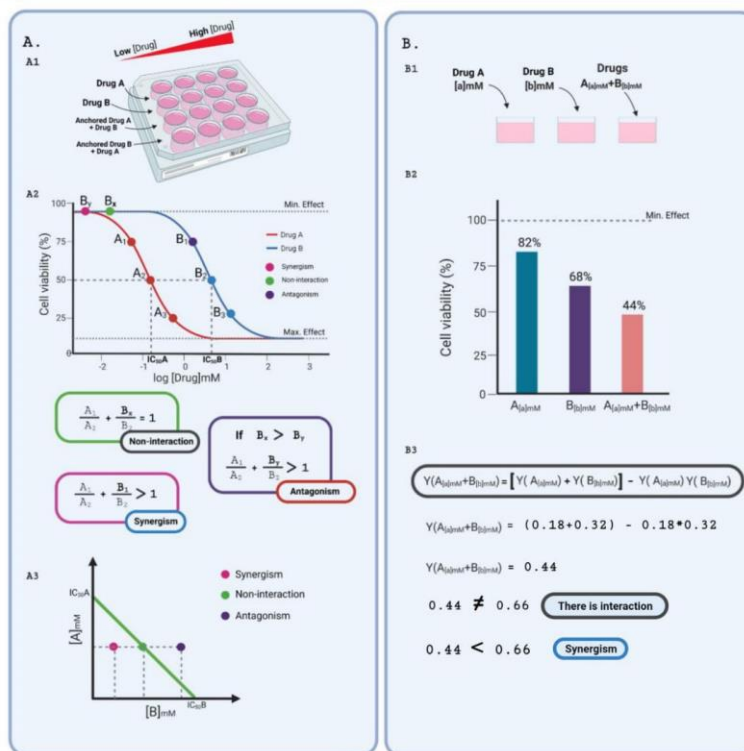
Figure 3 Summary of the two main mathematical approaches used to determine drug interactions, from which several other models were derived. **(A)** Loewe's additivity method. The A1-Typical experiment design employed in studies that apply Loewe's model. The drugs under evaluation (A and B in the picture) are individually administered to the cell culture in crescent concentrations. Also, dose-effect curves are drawn for an anchored (non-varying concentration) drug plus a varying drug. A2-Hypothetical dose-response curves for drugs A and B that aim to decrease cell viability, for example, chemotherapy, meeting the principles of drug equivalence, a pharmacodynamic similarity, constant potency ratio. Drug equivalence relies on the existence of doses that produce equivalent effects. In the figure, doses A1 and B1 are equivalent, as well as doses A2 and B2, and doses A3 and B3, as they produce the same percentage of reduction in cell viability, as the measured effect. Pharmacodynamic similarity is demonstrated by the same minimal and maximal effects reached by both drugs, and similar slope of the curves. Finally, the constant potency ratio is shown by the constant ratio observed between drug concentrations that produce the same effects. In simple words, A1/B1 = A2/A2 = A3/B3. Finally, combination index calculations originate dots throughout the graph representing the concentration of compound B required to achieve half-maximal effect in different combination scenarios: additive, synergic or antagonistic interactions. A3-An isobologram drawn from IC$_{50}$ concentrations of compounds A and B represent, for an anchored concentration of drug A, varying concentrations of B hypothetically required to produce the half-maximal effect in combination with A. Interactions are interpreted as additive (when the null model applies), synergic (when the concentration of B required for half-maximal effect is lower than expected), and antagonistic (when the concentration of B required for half-maximal effect is higher than expected). **(B)** Bliss's independence method. B1-Typical single-dose experiment design used to evaluate drug interactions applying Bliss's independence model. Drugs A and B are used at fixed concentrations, either individually or combined. B2-Histogram showing hypothetical cell viability measurements following treatments with drugs A and B drugs A and B that aim to decrease cell viability, for example, chemotherapy at fixed concentrations. B3-Mathematical interpretation of the hypothetical results represented in B2. As the action of both drugs is not independent (observed combined effects are different (lower) from the expected product of both individual effects), an interaction is detected and categorized as synergism. Image created with BioRender.

2000 FDA-approved or experimental drugs, three potential drug combinations were identified that now could be prioritized for experimental testing. Another in silico approach was capable of prioritizing 73 combinations of 32 drugs with potential activity against SARS-CoV-2, from which 16 synergistic and 8 antagonistic combinations were found in vitro, showing the usefulness of in silico approaches in the combat of the novel coronavirus outbreak [121]. Among the interactions identified we may highlight the remdesivir and hydroxychloroquine combination that demonstrated strong antagonism, and the nitazoxanide and remdesivir combination with a strong synergistic interaction [121]. The nitazoxanide-remdesivir combination to treat COVID-19 patients is very promising from a clinical perspective because both drugs are FDA approved, and the concentrations with synergistic effect identified in vitro can be achievable in plasma and lung [121].

Conclusion

The multiple targeting strategies through drug combination therapy raises many challenges since each drug has different properties [6].

The search for combined drugs targeting complementary mechanisms requires a large-scale search of several possible target combinations, but can also reveal potential synergies or interactions between disease-relevant pathways, leading to a greater understanding of the disease biology [4]. Despite the constant effort to quantify the effect of the combination of compounds in biological systems, there is still no agreement on which approach should generally be used since Loewe, Tallarida, Hand, Bliss, and HAS methods have limitations and problems [98, 122]. Although, each model of study has a different mathematical interpretation of results that should be considered in the study of possible combinations. In silico methods are extremely useful to lower the number of combinations to be tested in vitro, since you can prioritize more promising combinations and predict drug combinations with more accuracy. ML algorithms have been widely applied to predict drug synergy in the treatment of several diseases.

Knowledge of the mechanisms and consequences of pharmacokinetic and pharmacodynamic drug-drug interactions is crucial to the development of therapies since treatments can have secondary pharmacologic characteristics. Thus, it is important to consider that these effects generally depend on the concentration of the drugs through the dose-response effect, by the binding to specific receptors and the activity of drug-metabolizing enzymes or transporters. These effects can also change through DGI and DDGIs since genetic variability can alter the response of an individual to treatment, highlighting the importance of identifying possible drug interactions in different individuals to accurate therapeutic approaches [28].

Finally, although combined therapy has made advances in studies of a vast number of diseases as previously mentioned, it is clever to remember that treatments, despite improving the quality of life of patients, are hardly curative and further studies are always needed. The combined therapies have the potential to play an essential role in still unexplored therapies of several diseases, but it requires knowledge about the properties of the drugs, the multi-targets to be treated, and the possible genetic polymorphisms to better predict pharmacokinetics and pharmacodynamics and potential interactions [68].

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SÚMULA CURRICULAR

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5.PUBLICAÇÕES

Artigos completos:

Arnaud-Sampaio, VF; Bento, CA; Glaser, T; Adinolfi, E; Ulrich, H; Lameu, C. P2X7 receptor isoform B is a key drug resistance mediator for neuroblastoma. Frontiers in Oncology, 2022.

Arnaud-Sampaio, VF; Rabelo, ILA; Ulrich, H; Lameu, C. The P2X7 Receptor in the Maintenance of Cancer Stem Cells, Chemoresistance and Metastasis. Stem Cell Reviews and Reports, 2020.

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