Protein kinase A mediates adenosine A_{2a} receptor modulation of neurotransmitter release via synapsin I phosphorylation in medulla oblongata cultured cells

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Abstract

Synaptic transmission is a *sine qua non* process for neuron physiology. Such precise process is enabled in part due to modulation of neurotransmitter release. Adenosine is a putative synaptic modulator of neurotransmitter release in the Central Nervous System. The present study focuses on the modulation of neurotransmission by adenosine A_{2a} receptor as well its signaling intracellular pathway in the medulla oblongata cultured cells. Here, we provided evidence that adenosine A_{2a} receptor agonists, CGS21680, increases neurotransmitter release, in particular, glutamate and noradrenaline and such response is mediate by protein kinase A activation, which in turn increased synapsin I phosphorylation. These findings ascribed the *modus operandi* of A_{2a}R modulating neurotransmitter release in cultured cells from medulla oblongata cell of Wistar rats and suggest that protein kinase A mediates adenosine A_{2a} receptor modulation of neurotransmitter release via synapsin I phosphorylation.

Key words: Adenosine A_{2a} receptor – Protein kinase A – Synapsin – Medulla oblongata - Neuromodulation

Introduction

The complexity of the functions carried out by the nervous system relies on the ability of neurons to communicate with each other in an appropriate way. Such communication is enabled in part by the efficacy of synaptic transmission in regulating physiological processes. Neurotransmitter (NT) release occurs through the very complex process of exocytosis, which is subject to modulation by several factors, such as the presence of NT or neuromodulators in the synapse. This process, at the presynaptic level, is tune regulated by auto- and hereto- receptors which, by way of a negative or positive feedback mechanism, can adjust the exact amount of released NT for the information to pass to postsynaptic elements (Langer 2008). Many presynaptic receptors are able to modulate NT release (Ribeiro et al. 1996; Marchi and Raiteri 1996; Kato and Shigetomi 2001), by direct physical interactions, such as receptor-receptor interaction, as the case of G protein-coupled receptor heterodimerization (Fuxe et at. 2008), or it may involve intracellular signaling pathways, such as kinase protein activation (Fresco et al. 2004). Moreover, the coexistent of more than one NT which are simultaneously released from the same nerve endings, in particular purines, strongly suggests a modulatory role for adenosine and ATP as neuromodulators in the NT release (Dunwiddie 1985; Ribeiro and Sebastião 1987).

Adenosine is released by most cell and acts as a homeostatic molecule in the nervous system where it influences NT release (Ribeiro et al., 2002). Adenosine A_{2a} receptor ($A_{2a}R$), a G_s protein-coupled receptor, is widely expressed in the brain. Many evidences suggests that $A_{2a}R$ activation facilitates NT release in different neurotransmission systems, such as glutamate (GLU) and noradrenaline (NA) in the nucleus tractus solitarius (Castillo-Melendez et al. 1994; Barraco et al. 1995) as well γ-aminobutyric acid, GLU, acetylcholine and serotonin in hippocampus interneurons (Cunha and Ribeiro 2000; Cunha et al. 1995; Rebola et al. 2002; Okaba et al. 2001).

G_s protein-coupled receptor activation increases cAMP levels. Second messenger activation is implicated in mediating different aspects of short- and long-term changes in synaptic transmission (Leenders and Sheng 2005). Furthermore, activation of protein kinases in presynaptic terminals, in particular a classical cAMP target, protein kinase A (PKA), is correlating with increased transmitter release (Capogna et al. 1995; Trudeau et al. 1996; Trudeau et al. 1998). Nevertheless, a new cAMP receptor was identified, exchange protein activated by cAMP (EPAC) (De Rooij et al. 1998; Kawasaki et al. 1998). Since then, EPAC-mediated modulation of secretory mechanisms was reported in melanotrophs (Sdej et al. 2005), the crayfish neuromuscular junction (Zhong and Zucked 2005), as well as cortical neurons (Huang and Hsu 2006).

Synaptic vesicle (VS) release depends on an accurate event sequences, including NT transporting to VS, traffic to action zone, docking, fusion and endocytosis. In addition, neurotransmitter release also depends on several specialized proteins which control different steps leading to exocytosis. Synapsin (syn) is major SV-specific phosphoprotein which play multiple roles in several stages of synaptic transmission, including vesicles clustering, maintaining the reserve pool, vesicles delivery to active zones, and synchronizing neurotransmitter release events (Bykhovskaia 2011). These processes are modulated via a dynamic phosphorylation/desphophorylation cycle which involves several kinase, such as PKA (Jovanovic et al 2001).

The knowledge of these interactions and the detailed comparative analysis of their mechanisms may therefore be relevant for a better understanding not only of the receptor-mediated secretory mechanism modulation, but also of the neurotransmitter release physiology and it disorders, which might be represent suitable targets for pharmacological intervention by exogenous compounds.

Here, we were interested in the evaluation of intracellular signaling pathway to the A_{2a}R-mediated facilitation of neurotransmitter release in cell cultured from medulla oblongata of Wistar rats. We show that application of the A_{2a}R agonist CGS21680 (CGS) increases synapto-pHLuorin (SpH) and NADH fluorescence as well glutamate and noradrenaline levels in working solution. Only PKA pathway inhibition was able to inhibited CGS-mediated GLU and NA increasing levels. Moreover, CGS induced an increasing in synapsin I ($serine_9$) (syn I ser_9) phosphorylation and H89 was capable to abolish this response. Whereas no change was observed in the CGS-induced GLU and NA release when the cells were treated with EPAC inhibitor. Therefore, our results suggests that PKA-mediated A_{2a}R modulation of GLU and NA release in cell cultured from medulla oblongata of Wistar rats through synapsin I phosphorylation.

Method

Drugs and solution.

Brefeldin A, CGS21680, H89, GDH and NAD^+ (Sigma, USA); ZM241385 (TOCRIS, USA) and N^6 Benzoyladenosine-3',5'- cyclic monophosphate (6Bnz) and Chlorophenylthio-2'-O-Methyladenosine-3'5'-cyclic monophosphate (8pCPT) (BioLog, Germany). Working solution (WS) is composed of (mM) 140 NaCl, 5 KCl, 5 $ClCa^{2+}$, 1 $MgCl^2$, 10 HEPES/NaOH and 20 Glucose, pH 7.3 was used for all experiments unless otherwise indicated.

Dissociated dorsomedial medulla oblongata cell culture.

All the procedures and protocols were performed in accordance with The Institutional Guidelines for Animal Experimentation (Protocol 114/2010). Dorsomedial medulla oblongata were dissected from one-day old Wistar rats and prepared as previously described by Kivell and coauthors (2001). Briefly, cells were dissected out and dissociated in cold isotonic salt solution, pH 7.4. Cells were suspended in Neurobasal A media (Invitrogen, USA) supplemented with $_{L}$-glutamine (250µmol/L, Sigma), glutamax (250µmol/L, Gibco, USA), B27 (2%, Gibco) and gentamicin (40mg/L, Gibco). Viable cells were counted and plated on poly-$_{D}$-lysine-coated culture 12-well plates (TTP, USA) for neurotransmitter dosage assays, 96-well plates for in-cell western assay and confocal dish (Corning, USA) for image experiments at the concentration of 1800 cells/mm^2. Cultures were kept in a humidified incubator with 5%CO_2 and 95% air, at 37ºC, for 7 days. On the seventh day the medium was replaced by working solution and the cell culture submitted to pharmacological treatments.

Supereclipticˆ Synapto-pHLuorin transfection

To monitor NT facilitates release we used SpH, a reporter in which the pH-sensitive super-ecliptic pHluorin is fused to the intravesicular vesicle protein synaptobrevin2 (Miesenböck et al. 1998). Cells were transfected after 7 days *in vitro* using Lipofectamine LTX in OPTMEM according to the manufacturer's instructions (Invitrogen, Paisley, UK).

One day after transfection, cell in working solution was analyzed by means of time series image showing the SpH response during stimulation. Images were performed with a Zeiss LSM 510 Axiovert, the 488nm line of argon laser and 364nm Intreprize UV light to excite SpH. Fluorescence collection was done through a 40x oil numerical aperture lens using 485 and 385nm band-pass filter, a small adaptation of method previously described by Miesenböck and coauthors (1998).

Determination of glutamate and noradrenaline contents from work solution.

Glutamate and noradrenaline contents were determined by HPLC as previously (Mazzacoratii et al 1990; Ferreira et al 2005), with some modifications. Briefly, the chromatographic system consisted of a Shimadzu liquid chromatograph, model LC10A, an electrochemical detector, model L-ECD-6A, fluorescent detector, model RF535 (Shimadzu, Japan) and a 5µm particle size C18 supelcosil reversed phase column (4.6 x 250mm) (Supelco, USA). The HPLC was isocratically operated for both molecule analyses.

For the analysis of GLU, the mobile phase consisted of 0.05 M sodium citrate, 15% methanol, pH 5.95 flowed at a rate of 1.0 ml min^{-1} excitation and emission wavelengths of the detector were 350 and 460 nm, respectively. Derivatization process was carried out to produce fluorescence, briefly, sample was mixed with OPA/BME solution (27mg *o*-phtalaldehyde, 1mL methanol, 9mL sodium tetraborate pH 9.3 and 5µL β-mercaptoethanol) for 2min and then the derivatized sample was injected.

For the analyses of noradrenaline, the mobile phase consisted of 0.02 M dibasic sodium phosphate; 0.02 M citric acid, 0.12 mM EDTA, 556 mg 1^{-1} heptane-sulfonic acid, 2% methanol, pH 2.6 flowed at a rate of 0.5 ml min^{-1} with detector potential adjusted to -0.80 V.

The concentration of glutamate and noradrenaline released from cell culture was defined as the difference between the concentration after stimulation (treated) and before stimulation (control).

Measurement of the extracellular Glutamate concentration.

Glutamate increasing levels were detected using enzymatic assay (Bezzi et al 1998). In the presence of GLU and β-nicotinamide adenine dinucleotide (NAD^+), L-glutamic dehydrogenase (GDH) produces α-ketoglutarate and NADH, a product which fluoresces when excited at 360 nm. Images were performed with a Zeiss LSM 510 Axiovert, the Ultraviolet (UV) excitation was provided by Interprise UV light (Coherent, EUA) and detected through 40x oil numerical aperture lens. Fluorescence emission was collected through a dichroic mirror (510DRLP, Zeiss, Germany) and filtered with a 515EFLP filter (Zeiss, Germany). Cells were bathed in working solution and supplemented with GDH (50U/mL), NAD^+

(2mM) at ~23^{o}C. Background subtraction of the fluorescent signals was carried out by subtracting values recorded from the cells working solution lacking GDH and NAD^{+}. Data were expressed as dF/Fo, where Fo represents the fluorescence level of the optical field before cell treatment, and dF represents the change in fluorescence.

Synapsin I phosphorylation throughout in-cell western assay (ICW).

To analyze synapsin I phosphorylation, we used the in-cell western assay adapted from the protocol used by Chen and coauthors (2005). After treatments, the cells were fixed with 3.7% paraformaldehyde for 20 minutes. Following fixation, the cells were permeabilized by 5 washes with 0.1% Triton-X in PBS. Blockade were performed with 2% NGS, 4% BSA, 0.2% Triton-X in PBS for 30 minutes. The primary antibodies against synapsin I (Synaptic Systems, Germany) and synapsin I (ser_9) (Abcam, UK) diluted 1:150 in blocking solution and incubated overnight at 4°C with gentle shaking. The secondary antibodies goat anti-rabbit IRDye 800CW (LI-COR Biosciences, Germany) and donkey anti-mouse Alexa-Fluor 680 (Invitrogen, USA) were diluted 1:500 and 1:200 in blocking solution respectively and incubated for 1 hour at room temperature. The plates were imaged by scanning simultaneously at 700 and 800nm with an Odyssey Infrared Imaging System (LI-COR Biosciences, Germany). The datas were normalized by calculating the relation between the syn I ser_9 phosphorylated protein and syn I levels.

Data analysis.

Images were analyzed with Image J (NIH) and statistical analysis was performed using One and Two-way ANOVA following Bonferrini's or Dunnett's *post hoc* test using the program Prism from GraphPad Software (San Diego, CA).

Results

CGS-induced SpH fluorescence increase.

$A_{2a}R$ activation has been shown to produce neurotransmitter releasing facilitation in several areas of CNS (Popoli et al. 1995; Okaba et al. 2001; Ochi et al. 2000; Scislo and O'Leary 2006). To test whether $A_{2a}R$ stimulation may be facilitates NT release in cells from medulla oblongata, we transfected them with SpH, a biological sensor to analyze SV release. Although we found more than one cell transfect *per* plate, only one cell was analyzed. Upon CGS treatment, SpH fluorescence increased in the cell analyzed. To test whether CGS treatment evoked SpH fluorescence increase, we treated the cell with an $A_{2a}R$ antagonist ZM and

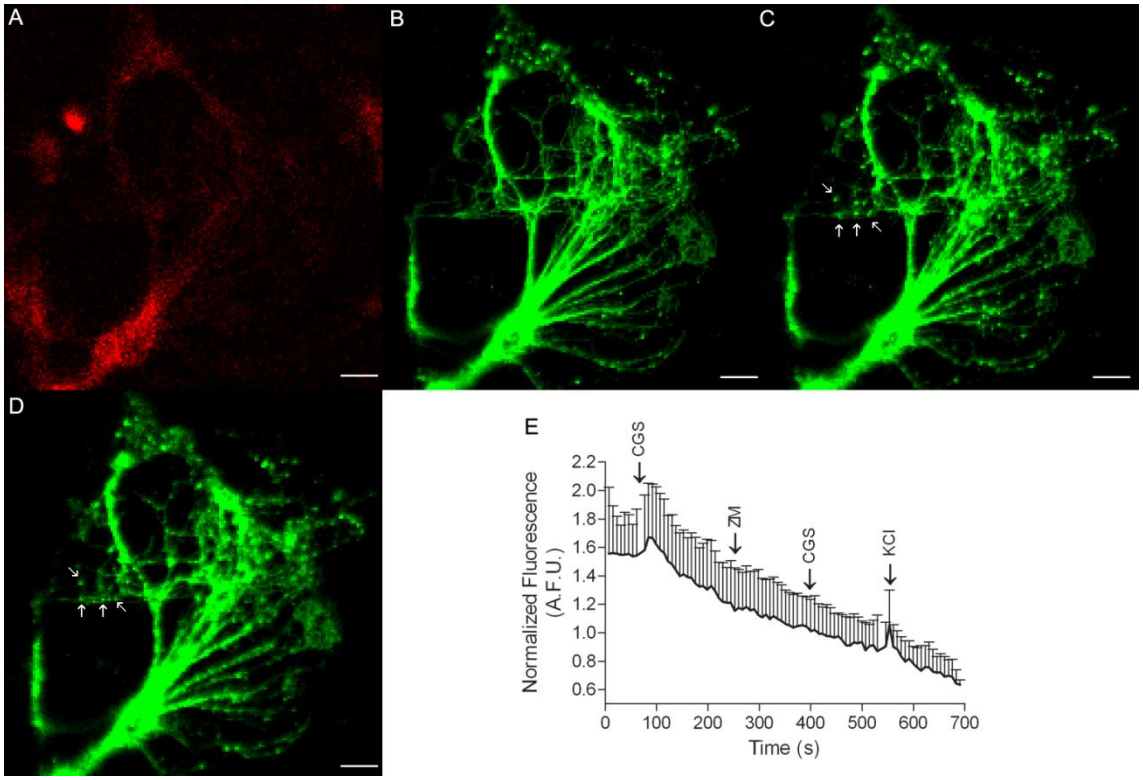

Figure 1: Effect of CGS21680 treatment on SpH fluorescence in medulla oblongata cells. (A), cell expressing SpH under 385 nm excitation. (B), SpH fluorescence under 485 nm excitation before treatments. (C), after CGS21680 stimulation. (D), after KCl stimulation. The scale bar is 10µM and arrows mark some points of registration which increased SpH fluorescence after CGS (5µM) treatment and KCl (90mM) depolarization. (E), the SpH normalized fluorescence is represent as ratio 485/385nm. As expect, it was a gradual time-dependent decay of SpH fluorescence. Upon CGS treatment, SpH fluorescence increased, on the other hand, after ZM treatment, CGS did not change SpH fluorescence. At the end, KCl treatment induced an increase in SpH fluorescence confirming that the cell remains responsible to stimulation. Data are expressed as means ± SEM, (n=4).

then stimulating again with CGS and no changes in the fluorescence basal line were observed. To confirm whether ZM was capable to abolish CGS-induced SpH fluorescence increases, we added KCl at the end of the assay. KCl increased SpH fluorescence suggesting that the cell remain responsible to stimulus (Figure 1).

CGS-induced NADH fluorescence increase.

When GDH and NAD^+ were added to the working solution, stimulation of the cells induced a transient increase in the NADH fluorescence. After fluorescence signal line stabilization we set the first ten points as basal line and then, 5µM of CGS were added. CGS induced an increasing in GLU extracellular levels which caused an elevation in NADH fluorescence (dF/Fo) (Figure 2). To confirm that the fluorescence signal originates from the activity of extracellular GDH, we performed identical procedure lacking one of its components. When either GDH or NAD^+ was omitted from the working solution, no fluorescence changes were observed after CGS treatment (data not shown).

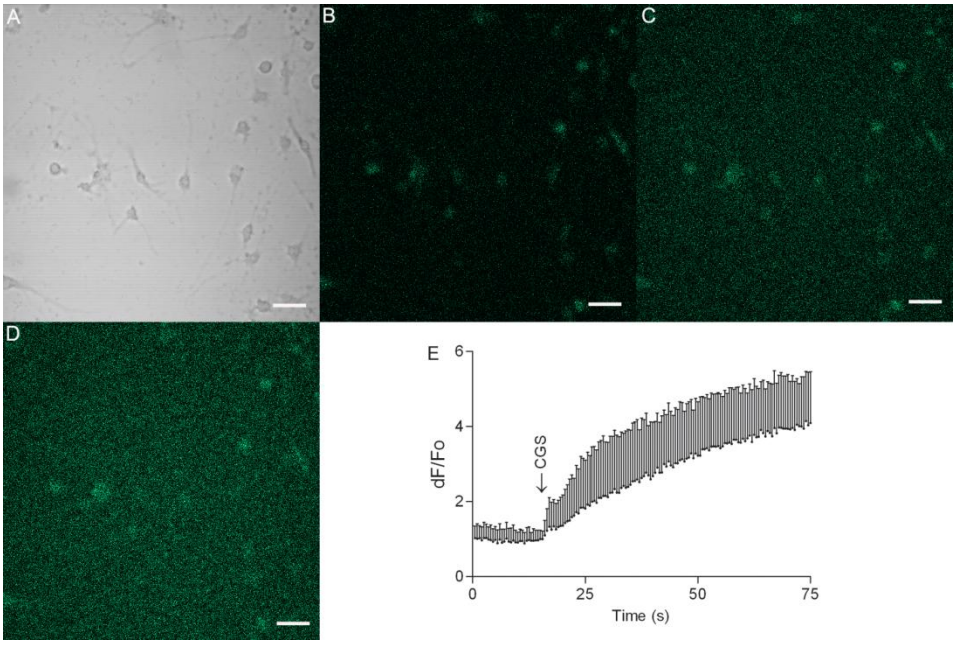

Figure 2: Elevation in NADH fluorescence in response to CGS21680 treatment in cultured cells from medulla oblongata. (A), cells without UV excitation. (B), under UV excitation before treatment. (C and D), change in NADH fluorescence intensity during CGS (5µM) stimulation. (E), the graph representing the changes in NADH fluorescence ratio during CGS (5µM) stimulation. NADH fluorescence value corresponds to the ratio dF/Fo, where Fo represents the fluorescence level of the optical field before treatment, and dF represents the change in fluorescence. Scale bar is 50µm and data are expressed as means ± SEM, (n=4).

Time and concentration characterization of CGS-mediated GLU and NA increased levels in working solution.

Evidences suggesting that $A_{2a}R$ activation facilitates glutamate and noradrenaline release in neurons from the nucleus of tract solitary (NTS) of rats (Castillo-Merendez et al. 1994; Barraco et al. 1995). To determinate whether $A_{2a}R$-mediated facilitation of GLU and NA release in cultured cell from medulla oblongata, we determinate GLU and NA contents in the work solution through HPLC and also carried out CGS time-course and concentration-response (0.1-100µM) assays. We observed more pronounced response at 5s for both neurotransmitters in response to CGS treatment and a CGS EC_{50}/standard error = 4,88µM/0,026 and 4,25µM/0,038 for GLU and NA respectively. Therefore, 5µM of CGS for 5s was used as $A_{2a}R$ agonist treatment in the following GLU and NA content determination by HPLC (data not shown).

PKA-mediated $A_{2a}R$ modulation of GLU and NA increase levels in work solution.

To test which cAMP effector is involved with a transient increase in GLU and NA levels in work solution of medulla oblongata cultured cells evoked by $A_{2a}R$ stimulation, we performed pharmacological treatments activating and inhibiting $A_{2a}R$, PKA and EPAC. We first observed that CGS treatment was able to increased GLU and NA levels in WS. When added with CGS, an $A_{2a}R$ antagonist (ZM), it was able to abolish GLU and NA increase levels mediated by $A_{2a}R$ activation. The profile of GLU and NA

increased levels in response to $A_{2a}R$ stimulation was strikingly similar (Figure 3 A and B). $A_{2a}R$ stimulation increases adenylyl cyclase activity, therefore raising cAMP concentration, which in turn might activated different effectors. To provide direct evidence for PKA-mediated CGS modulation of GLU and NA increasing levels, we next treated the cells with a PKA inhibitor (H89) and CGS. Under this condition, we did not observed a CGS-induced GLU and NA increases levels (Figure 3 C and D). To determine whether other cAMP effector also be involved in CGS-induced GLU and NA increases levels, we inhibited other recently describe cAMP target, EPAC. The addition of an EPAC inhibitor (BFA) with CGS did not change the response observed with only CGS treatment (Figure 3 C and D). To further characterize the mechanism of PKA-mediated $A_{2a}R$ modulation of GLU and NE increasing levels, we treated the cells with CGS and a PKA activator (6Bnz). 6Bnz enhanced CGS-mediate GLU and NA increase levels in WS. Although, only PKA inhibition was capable to prevent CGS-induced GLU and NA increases, we also tested a treatment with CGS and an EPAC activator (8pCPT) which did not change the response observed in CGS treatment (Figure 3 E and F). These observations strongly support the notion that PKA-mediated CGS modulation of GLU and NA increases levels in medulla oblongata cultured cells.

CGS21680 modulated synapsin I phosphorylation.

Synapsin I is the most abundant isoform in mature neurons of mammals (Ferreira and Rapoport 2002) and acting controlling SV transitions from the reserve pool to the readily releasable pool at active zone through a phosphorylation-dependent regulation of the link between SV and actin filaments (Bonanomi et al. 2005). Nine phosphorylation sites have been identified in synapsin I and the site 1 ($serine_9$) was identified as a substract for PKA phosphorylation (Cesca et al. 2010). In-cell western assay has been employed for the study of protein phosphorylation with high sensitivity and precision (Aguilar et al. 2010). Therefore we use ICW to evaluated syn ser_9 phosphorylation in response to CGS treatment. To accurately analyse syn I phosphorylation, we measured syn I ser_9 phosphorylated and syn I fluorescence intensity and normalized the datas dividing syn I ser_9 by syn I intensities.

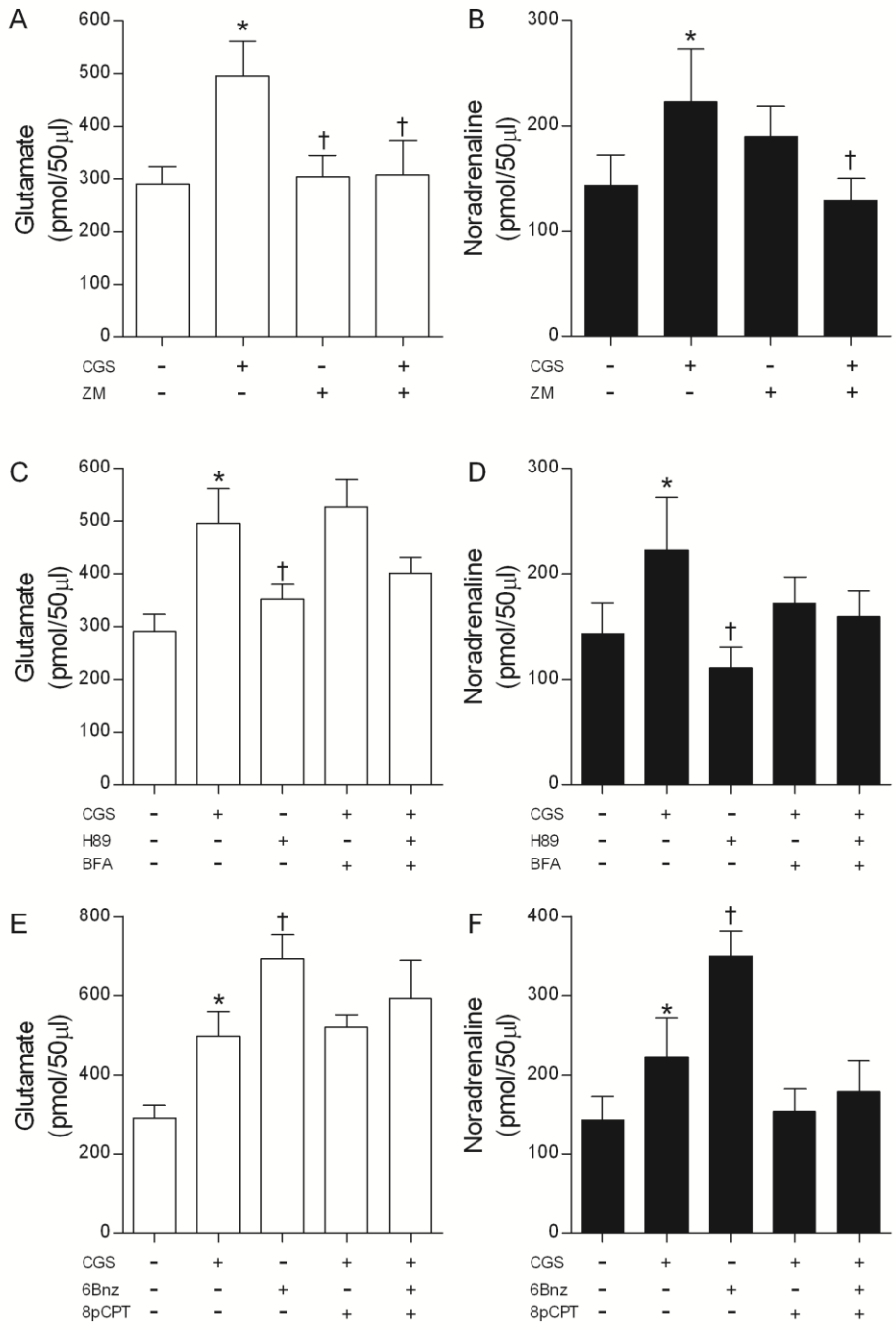

Figure 3: A_{2a}R-mediated GLU and NA increases in medulla oblongata cultured cells are associated with PKA activation detected by HPLC. (A, C and E) Glutamate, (B, D and F) Norepinephrine levels in response to treatments of agonist and antagonist A_{2a}R, as well as, activators and inhibitors of PKA and EPAC. CGS-induced GLU and NA increased levels in WS. When added to CGS, ZM abolish GLU and NA increases mediated by A_{2a}R agonist (A and B). H89 inhibited CGS-induced GLU and NA increases (C and D). The CGS-induced GLU and NA increasing levels were not inhibited by BFA (C). When a PKA activator 6Bnz was added with CGS, a more pronounced increasing of GLU and NA levels was observed when compared to CGS treatment. No changes were observed in EPAC activator treatment (8pCPT) when added with CGS in both GLU and NA levels when compared to CGS treatment alone (E and F). The data are represents by mean ± standard deviation (n=4), * $p< 0.05$ when compared with control, † $p<0.05$ when compared with CGS treatment. One-way ANOVA following Bonferroni's *post-hoc* test was employed in the statistical analysis.

To evaluate the temporal relationship between syn I phosphorylation and CGS treatment, we treated the cells with 5μM of CGS for 5, 10, 20, 40, 80 and 160 seconds. Interestingly, we observed an increasing in syn I phosphorylation only at 5s (data not shown).

PKA-mediated synapsin I ser$_9$ phosphorylation occurs in response to CGS treatment.

To characterize the PKA-mediated CGS-induced synapsin I phosphorylation, we treated the cells with $A_{2a}R$ agonist and antagonist, as well as PKA inhibitor and activator, at the same concentration used in neurotransmitter dosage assays (HPLC) for 5s. As expect, CGS induced an increasing in syn I phosphorylation when compared to control. ZM treatment did not change syn I phosphorylation levels when compared to control, although it was able to prevent CGS-induced syn I phosphorylation increase when the cells were treated with both drugs simultanelly. Inhibition of PKA by treatment with H89 abolished syn I phosphorylation increase mediating by CGS treatment. We did not observe an increasing in syn I phosphorylation when the cells were treated with CGS and PKA actvator (6Bnz) as compared to CGS treatment (figure 4). It is interesting to note that syn I expression was found in undifferentiated astrocytes, which declined significantly during differentiation in culture (Maienschein et al. 1999) and no significant expression of syn I protein was detected in astrocytes *in vivo* (Cahoy et al. 2008) suggesting that syn I fluorescences is neuron-specific.

Discussion

To our knowledge this study is the first to evaluate neurotransmitter release facilitation taking account adenosine A_{2a} receptor stimulation, intracellular signaling and proteins involved in synaptic vesicle release in cultured cells from the medulla oblongata of Wistar rats.

Although different receptors are involved in receptor-mediated neurotransmitter release facilitation (Langer 2008), results have demonstrated herein may be of relevance for the understanding of NT release modulation by a GPCR since our pharmacological target was $A_{2a}R$, a G_s GPCR. Moreover, because the present study was performed in cells from medulla oblongata, the data might clarified possible intracellular mechanism to $A_{2a}R$-mediated neurotransmitter release in the NTS of living rats (Barraco et al. 1995; Castillo-Melendez et al. 1994). In addition, as we analyzed the influence of two cAMP effectors (PKA and EPAC) in this phenomenon, as well

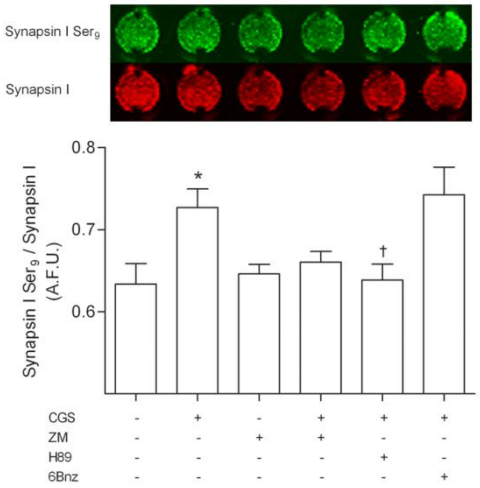

Figure 4: A_{2a}R activation induced an increasing in synapsin I (ser_9) phosphorylation in medulla oblongata cultured cells detected throughout in-cell western assay. Signals from anti-synapsin I (ser_9) phosphorylated apper as green fluorophores, while anti-synapsin I signals appear as red fluorophores. The datas were normalized dividing synapsin I (ser_9) phosphorylation by sinapsin I fuorescences and the values expressed as arbitrary fluorescence units. The cells were treated with CGS, ZM, H89 and 6Bnz for 5s. CGS exposure induced a increasing in syn I (ser_9) phosphorylation when compared to control, as well as H89 was capable to inhibiting this response. The values are represents by mean ± standard deviation (n=6), * $p< 0.05$ when compared with control, † $p<0.05$ when compared with CGS treatment. One-way ANOVA following Bonferroni's *post-hoc* test was employed in the statistical analysis.

as a SV phosphoprotein which has a specific site for PKA phosphorylation, suggesting that our results might be related to the intracellular *modus operandi* of A_{2a}R modulation of NT release in medulla oblongata cultured cells.

Adenosine is the predominant presynaptic modulator of transmitter release in the central system nervous (Dunwiddie 1985). Under physiological conditions, synaptic adenosine is formed from vesicular release of ATP followed by its extracellular degradation to adenosine via a cascade of ectonucleotidases and intracellular production followed by extracellular transport (Jacbson and Gao 2006). The vesicular release of ATP, not from neurons but from astrocytes, has been identified as a major source of synaptic adenosine (Pascual et al 2005). Furthermore, due to its interactions with several neurotransmission systems and other neuromodulators, adenosine can be regarded as a "master regulator" to integrate and fine-tune of several neurotransmitter systems in the CNS (Sebastião and Ribeiro 2009). Adenosine may modulate synaptic transmission in two opposite manners, inhibiting via A_1 receptor or enhancing via A_{2a}R. Therefore, adenosine, in particular A_{2a}R activation, has been an important experimental model to evaluate neurotransmitter release.

Interestingly, Correia-de-Sá and coauthors (1991) showed that both receptors (adenosine A_1 and A_{2a} receptors) can be expressed at the same nerve terminal. Moreover, evidences suggesting cross talk interaction between adenosine A_1 (A_1R) and A_{2a} receptors in the hippocampus, where A_{2a}R induced activity attenuation of A_1R which enhancing the synaptic transmission (O'Kane and Stone 1998; Lopes et al. 1999 and 2002; Cunha and Ribeiro 2000). Therefore, the question of how adenosine is able to

tonically modulate neurotransmitter release has tackled by several studies to attempt to understanding the mechanisms involved in such modulation.

In order to rule out the possibility that $A_{2a}R$ stimulation increases basal NT release, we transfected cells of medulla oblongata with SpH which has been used as an important biological sensor to analyze SV release mechanism (Sankaranarayanan and Ryan 2000; Atluri and Ryan 2006; Moulder et al. 2007). CGS treatment enhanced SpH fluorescence, therefore this observation may reflect the possibility that $A_{2a}R$ stimulation acts as a neurotransmitter release modulator in cells from medulla oblongata.

It is plausible considering that the CGS-mediated SpH fluorescence increase was not mediated by CGS directly. Since other neurons or glia cells might be also activated by CGS and, therefore, stimulates the cell analyzed (Boison et al. 2010).

According to Castillo-Merendez and colleagues (1994), as well Barraco and coauthors (1995) studies, $A_{2a}R$ stimulation facilitates glutamate and noradrenaline release in the nucleus of solitary tract of rats which is located in the medulla oblongata. Therefore, these results corroborate to ours finds where in cell culture from dorsamedial medulla oblongata, CGS treatment increased GLU and NA levels in working solution. Interestingly, $A_{2a}R$-modulated glutamatergic and noradrenalinergic neurotransmission is not an exclusive phenomena of the brain area studied from the present work, *e.g.*, CGS enhanced [^{3}H]-norepinephrine release from postganglionic sympathetic nerve terminals in rat tail artery (Fresco et al. 2004), as well as $A_{2a}R$ activation facilitate glutamatergic transmission in hippocampus (Constenla et al. 2011) and striatal neurons (Popoli et al. 1995), suggesting that this receptor exerts a fine-tuning modulation of glutamate and noradrenaline release in the nervous system.

Although our aim was CGS-induced GLU release mediating by neurons (Castillo-Merendez et al. 1994), it is possible that in cultured cell at least a small part of GLU was also released from astrocytes in an indirect manner. This is partly due to $A_{2a}R$ are also expressed in NTS glial cells (Pickel et al. 2006), as well astrocytes are relationship to glutamate homeostasis including in gliotransmitter release of glutamate, ATP and $_D$-serine, as know as tripartite synapse (Boison et al. 2010).

In addition, evidences that $A_{2a}R$ may activates different intracellular transduction pathways via cross talk, *e.g.*, protein kinase C (O'Kane and Stone 1998; Lopes et al. 1999). However, the major signal transduction pathway involved in response to its activation is the cAMP signal which in turn is mediated by adenylyl cyclase increasing activity (Fredholm et al. 2011). Thus, we initially aimed our pharmacological treatments in this signal transduction pathway.

Initially, all effects of cAMP were attributed to PKA activation, however, two other targets may be involved in mediating cAMP responses, a cAMP-regulated ion channels and EPAC (Tanken and Aandahl 2004). Although cAMP may regulate ion channels, only PKA and EPAC-mediated NT release facilitation were described (Castelluci et al. 1980; Chavez-Noriega and Stevens 1994; Gekel and Neher 2008). Therefore, we aimed on the role of these two cAMP effectors (PKA and EPAC) in NT release facilitation mediate by $A_{2a}R$ activation. Our results showed that only PKA inhibition was capable to prevent CGS-induced NT release facilitation. Moreover, a PKA activator (6Bnz) enhanced GLU and NA increase levels when compared to CGS treatment. Taken together these results discussed above plus the fact that both EPAC inhibitor and activator did not change CGS-induced NT release facilitation, we therefore suggest that PKA-mediated CGS induces NT release facilitation in cell culture from medulla oblongata.

One possible interpretation is that PKA through anchoring via A kinase anchoring proteins (AKAPs) is located close of the GPCR providing a fast and specific cAMP target (Taskén and Aangdahl 2004). PKA is an isozyme consisting of two regulatory and two catalytic subunits. The catalytic subunits are responsible for catalyzing the phosphoryl transfer reaction (Murray 2008). It is interesting to note that protein interactions within the release *apparatus*, such as protein phosphorylation or dephosphorylation, may be one mechanism by which cellular events modulate synaptic transmission (Leenders and Sheng 2005). Furthermore, PKA phosphorylation of the proteins associated with regulated exocytosis has been an important event in the process of cAMP-regulated exocytosis in several systems (Hirling and Scheller 1996; Jovanovic et al. 2001; Chheda el al. 2001; Risinger and Bennet 1999).

Synaptic vesicles are typically organized in clusters at presynaptic terminal and those clusters are distinguished based on their location and function. The clusters closest to active zone are released first and are coined as the readily releasable pool (RRP). A larger cluster of SVs is situated more distal to the release site and comprises the reserve pool (RP), which replenishes the RRP during its stimulation-induced depletion. Since the RRP accounts for only about 1% of the total SVs in some synapse, while RP (~10-15%), the existence of a third, so called resting pool has been suggested. However, this pool does not actively contribute to neurotransmission under normal conditions, but only upon sustained intense stimulation (Rizzoli and Betz 2005). Although only synaptic vesicle in RRP is release at the synapse, the modulation of trafficking of VS from RP to RRP altering directly the availability of SV at active zone and therefore at least in part may facilitating the synaptic transmission.

We found that CGS increased syn I ser_9 phosphorylation and H89 treatment was able to abolish this response. These data strongly support that synapsin I phosphorylation by PKA might be the mechanism involved in $A_{2a}R$ activation mediate facilitation of glutamate and noradrenaline release. Once it was demonstrated throughout a proteomic analysis that a single SV has about eight synapsin proteins (Takamori et al. 2006) and the ability of syn I to bind to SVs and to actin filaments in a phosphorylation-dependent manner (Greengard et al. 1993; Cesca et al. 2010) led to the hypothesis that syn I serves as a regulator of trafficking of SVs between the RP to RRP and therefore, affecting the availability of SVs for exocitosis at active zone.

Although It has been reported that site 1 of syn I can be phosphorylated by PKA and Ca^{2+}-calmodulin-dependent protein kinase II (Chi et al. 2001), recent data indicated that syn I phosphorylation at site 1 is primarily mediate by PKA, which potentiates activity-dependent SV exocytosis via syn I ser_9 phosphorylation in hippocampal neurons (Menegon et al. 2006) and *Helix* pre-synapses of invertebrates (Fiumara et al. 2004). Furthermore, lack of syn I reduces the RRP of synaptic vesicles at inhibitory synapses in hippocampal cultured cells from syn I knock-out mice impairing synaptic transmission (Baldelli et al. 2007).

Collectively, our results suggest that $A_{2a}R$ activation enhancing glutamate and noradrenaline release via PKA-mediated synapsin I phosphorylation in cultured cell from medulla oblongata of Wistar rats.

Based on these results, we conclude that CGS21680 treatment increased glutamate and noradrenaline levels, and synapsin I phosphorylation, as well as PKA inhibition abolish these responses.

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