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investigação dos circuitos neurais mediando a esquiva ativa instrumental e a esquiva contextual não instrumental

investigation of the neural circuits mediating instrumental active avoidance and non instrumental contextual avoidance

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RESUMO

Título: Investigação dos circuitos neurais mediando a esquiva ativa instrumental e a esquiva contextual não instrumental

Mamíferos, incluindo roedores, mostram uma ampla gama de comportamentos defensivos como forma de lidar ativamente, como comportamentos de esquiva, ou passivamente, como comportamento de congelamento ("freezing"). A resposta de esquiva é uma resposta aprendida na qual um indivíduo assume o controle em situações perigosas para lidar com ameaças. Uma forma de esquiva investigada é a esquiva ativa sinalizada, na qual os indivíduos são treinados a se esquivar de um estímulo aversivo fazendo uma tarefa aprendida em resposta a apresentação de uma pista previamente associada ao estímulo aversivo. Foi evidenciado que o CPFdm desempenha um papel importante na codificação da aquisição e expressão de congelamento, bem como nas respostas de esquiva. No entanto, sua contribuição para a aquisição e expressão do comportamento de esquiva não é clara e os circuitos neurais do processamento do CPFdm ainda não foram descobertos. Para resolver essa questão, desenvolvemos um novo paradigma comportamental, no qual o camundongo tem a possibilidade de "freeze" passivamente ao estímulo aversivo ou evitá-lo ativamente em função de contingências contextuais. Nessa primeira parte do projeto, estudamos o papel da via entre o CPFdm e a PAG na esquiva ativa sinalizada e sua relação com o congelamento. Nossos resultados indicam que (i) o CPFdm e a dl/lPAG são ativadas durante o comportamento de esquiva, (ii) a inibição optogenética dessa via bloqueou a aquisição de esquiva condicionada mas não alterou a resposta de congelamento. Uma forma não instrumental de esquiva, foi investigada, onde o indivíduo aprende a evitar o ambiente aversivo usando apenas pistas contextuais e exibindo comportamentos de avaliação de risco em relação ao ambiente aversivo. Nesta situação foi demonstrado que uma via específica septohipocampo-hipotalâmico-tronco encefalico está envolvida. Esta análise revelou que o núcleo pré-mamilar dorsal (PMD) deva estar criticamente envolvido na expressão de esquiva passiva. Nos analisamos como a manipulação do PMD e suas projeções para seus principais alvos influencia os processos de expressão e re-consolidação da esquiva passiva contextual. Nossos resultados mostraram que (i) uma via específica septohipocampo-hipotalâmico-tronco encefálico está envolvida em nosso paradigma de esquiva passiva. (ii) O silenciamento do PMD durante a exposição ao contexto prejudica tanto a expressão de esquiva quanto a reconsolidação de memória e que (ii) a inibição no nível terminal prejudica a expressão e a reconsolidação de memória tanto em dlPAG quanto em AMv. investigamos essas questiões com a análise imunoquímica de Fos, manipulações de circuitos neurais usando técnicas optogenéticas e fármacogenéticas.

Palavras-chave: Esquiva ativa. Esquiva passiva. Cortex préfrontal. Hypothalamo. Optogenética

ABSTRACT

Title: investigation of the neural circuits mediating instrumental active avoidance and non instrumental contextual avoidance

Mammals, including rodents show a broad range of defensive behaviors as a mean of coping actively, such as avoidance behaviors, or passively such as freezing behavior. The avoidance response is a learned response in which an individual takes control in dangerous situations to deal with threats. One form of avoidance that has been investigated is the signaled active avoidance, where individuals are trained to avoid an environment, and escape in response to a cue previously associated with an aversive stimulus. It has been emphasized that the dmPFC plays an important role in encoding freezing acquisition and expression as well as active avoidance responses. However the neural circuits of the dmPFC processing the expression and aquisition of both active and passive coping strategies are yet to be discovered. To adress this question, we developed a novel behavioral paradigm in which a mouse has the possibility to either passively freeze to an aversive stimulus or to actively avoid it as a function of contextual contingencies. We first investigated the role of the pathway between the dmPFC and PAG in signaled active avoidance, and its relation with freezing. Our results indicate that (i) dmPFC and dl/lPAG sub-regions are activated during avoidance behavior, (ii) and that the optogenetic inhibition of this pathway blocked the acquisition of active avoidance. A non-instrumental form of avoidance is also investigated where the individual learns to avoid the aversive environment using contextual clues only, and displaying risk assessment behaviors toward the fearful environment. It has been previously shown that in this situation, a circuit involving the septohippocampal-hypothalamic-brainstem pathway is involved. It also revealed that the dorsal premammillary nucleus (PMD) must be critically involved in contextual passive avoidance. We analysed how the manipulation of the PMD and its projections to its main targets influences the expression and re-consolidation processes of contextual passive avoidance. Our results showed that (i) a specific septohippocampal-hypothalamic-braintem pathway is involved in our passive avoidance paradigm. (ii) Silencing the PMD during context exposure impairs both avoidance expression and memory reconsolidation and that (iii) the inhibition at terminal level impairs the expression and memory reconsolidation in both dlPAG and AMv. Both parts of the project assessed these questions using Fos immunochemistry analysis, manipulations of neural circuits using optogenetic, and pharmacogenetic techniques.

Keywords: Active avoidance. Passive avoidance. Prefrontal cortex. Hypothalamus. Optogenetic.

Introduction

1) Active and Passive avoidance

a/ Fear defensive strategies

Depending on the environment, animals present a repertoire of defensive behaviors related to their survival needs. Indeed, animals adopt defensive strategies to protect themselves and/or their conspecifics against environmental dangers. Moreover, when the danger is escapable, more active defensive behaviors such as avoidance, escape and flight are adopted (Ramirez, et al., 2015; Blanchard and Blanchard, 1969). Adaptation includes selecting the appropriate defensive strategy taking into account its costs, the threat, and the context in which it occurs (Hofmann and Hay, 2018). As mentioned above, avoidance is one defensive strategy adopted when an individual is exposed to harm but has the possibility to put distance with the threat. However, under certain circumstances, for instance inescapable situation, individuals eventually adopt other defensive strategies, like freezing (LeDoux, 2012). Promising fields of research have been explored to study emotional coping strategies, and a large variety of paradigms have been developed in order to disentangle the circuits recruited in defenses responses of an individual to fear. The most complete study about defensive behaviors in rodents had been carried out by the Blanchards. The idea was to predict which defensive behavior would be selected depending on the different contextual and stimuli changes. An example of this grouping of tasks is the Mouse Defense Test Battery MDTB (Blanchard, et al., 2003; Blanchard, 2017). In these studies, numerous defensive responses in rodents exposed to threatful situations have been observed: flight, hiding, freezing, attack and risk assessment. An example of MDTB tests is a long oval runaway permitting to quantify escape behavior that can be modified and transformed to an unescapable arena to study the switch to freezing strategy. Indeed, freezing and avoidance has been one of the most studied defensive behaviors. Regarding freezing, some studies describe it as being a passive tonic immobilization (Blanchard and Blanchard, 1972; LeDoux, 2000), but other researchers argue that freezing is an active preparation state during which the organism gets ready to flight, avoid or fight (Gladwin, et al., 2016). It is why this passive response is

interesting to be compared with active behaviors like avoidance, in terms of brain circuits and behavior selection.

During threatening escapable situation, individuals usually demonstrate predictable goal directed behaviors. Indeed, there are two main categories of motor responses learned under negative reinforcement: escape behavior and risk assessment/avoidance behaviors. Escape behavior is a motor action performed by the animal to terminate an ongoing aversive stimulus. This behavior is negatively reinforced by the elimination of the unpleasant stimulus. For instance, a rat will flee the room if receiving a shock on an electrified floor. Fleeing to stop the shock is an escape behavior. One characteristic of flight behavior, in more naturalistic situations, is that the initiation of the movement is very sensitive to the distance separating the animal from the potential threat. For example, the rat needs to be quite close to the predator to elicit a flight response, it is the concept of threat imminence (Kim et al., 2013; Lowe et al, 2015). In learned tasks, escape behavior is converted into avoidance behavior by giving a signal before the aversive stimulus starts. In this case, avoidance represents complex motor actions learned by repetitive trials of conditioning paradigms (Moscarello and LeDoux, 2013). In innate situations, or open threatful environments, avoidance behavior would be the action to not approximate the localized threat, by scanning the environment with flat back approaches and oriented stretched postures (Dielenberg, et al., 2001; Blanchard, et al., 2003). Risk assessment behaviors (RA) are expressed as mentioned before in natural conditions (Blanchard, et al., 2003;), as for example the exposure to a cat or cat odor (Blanchard et al, 2005; Osada, et al., 2013). RA also correspond to the animal scanning of the environment to detect routes of possible hiding or escape (Ellard and Eller, 2009). In a recent study, these behaviors are proposed to be a good model to compare anxious behaviors in human (Blanchard et al., 2019).

These active defensive strategies are encountered various escapable situation that can be modeled using different types of experimental avoidance paradigms.

b/ Paradigms of avoidance learning

Most of the avoidance paradigm encountered in the literature are based on Pavlovian conditioning. It is the way to associate an aversive unconditioned stimulus (US), to a neutral conditioned stimulus (CS) which can be either an acute signal (Morgan and LeDoux, 1995), or the context itself (Baldi, 2004). The US presented can be of different natures, the most commonly used ones are mild electrical shocks both in humans (Low, et al., 2015) and rodents (Bravo-Rivera, et al., 2015; LeDoux, et al., 2017), however other types of US can be encountered, like air puffs (Moriarty et al., 2012), aversive odors (Osada, et al., 2013), or predators (live or robots) (Blanchard et al, 2005, Kim et al., 2015). In the literature, avoidance paradigms are usually divided in two types of study: the active and the passive avoidance studies. The next paragraphs will describe the different paradigms encountered in each type of study, introducing our choice of paradigm to study instrumental active avoidance, and non instrumental passive avoidance.

Instrumental Active avoidance paradigm

This project will first focus on the strategy of active avoidance, which consists on taking action to prevent harm. It is often studied using one-way or two-way active avoidance paradigms. In one-way active avoidance paradigms, only one of the two chambers of a shuttle-box is aversive (Gebhardt, et al., 2013) and associated with a shock presentation. In two-way active avoidance, both chambers can be aversive, therefore the behaviors expressed are less context dependent as compared to one-way avoidance paradigms. Two-way active avoidance paradigms can be either signalled by a stimulus such as a tone or a light, or unsignalled (Servatius, et al., 2016). In unsignalled (or Sidman) avoidance conditioning, the individual receives an aversive stimulus at fixed intervals, without any warning signal. In order to reset the timer to zero and cancel the shocks, a shuttle to the other side is required. However, unsignalled active-avoidance is very difficult to acquire in rodents, which is why signaled two-way active avoidance is preferred in our case. The two-way signaled avoidance (SigA) is a more complex paradigm that involves two forms of conditioning, the Pavlovian and the instrumental, which produce conflicting behavioral responses, and must be reconciled to ensure that the

individual responds adequately in order to avoid the aversive stimulus. Associative, or Pavlovian learning, is a simple and fundamental form of memory formation (Pavlov, 1927), where as described before, and individual associate and aversive unconditioned stimulus (US), to a neutral conditioned stimulus (CS). Instrumental, or operant conditioning, initiated by the behaviorist Skinner (Skinner, 1938), is the association of an action that will lead to a specific outcome when a motivational event is repeatedly displayed. This motivation, or reinforcement, to perform an action can be either positive or negative. the two-way sigA is and experiment that requires a shuttle-box separated into two compartments by a door or a hurdle. The animal learns to cross during the warning signal to anticipate the delivery of the unconditioned stimulus (US) (Ramirez, et al., 2015). Therefore, the two-way SigA is based on what is called the two-factor theory proposed by Mowrer (Mowrer, 1947) as the task reconciles the two principles of Pavlovian and instrumental learning.

In our case, we chose the two-way signal active avoidance paradigm, with the difference that the contextual contingencies demonstrate either escapable (opening of the door between the two comportments), or unescapable (the door stays closed) situations. The rationales of this choice will be described later in the introduction.

Non instrumental Passive avoidance paradigm

Passive avoidance, also labelled inhibitory avoidance refers to abstaining from entering a likely to be aversive environment (i.e. entering a footshock compartment). It is important to note that passive avoidance does not mean passive coping behaviors. Interestingly, while assessing the environment and integrating aversive cues the individual expresses a range of risk assessment behaviors (RA), that are likely to be opposite to the freezing state (Blanchard et al, 2003). Passive avoidance studies are of a strong importance for different reasons, as for instance investigating the neural circuits underlying the learning of ‘’what to not do’’. It is described in the step-down inhibitory avoidance paradigm, like deciding to step or not on an electrified platform where the animal had previously received a shock (Canto de Souza, 2016). The paradigm of contextual passive avoidance is also commonly used in innate threat exposure. The

animal is usually exposed to a predator (a cat, an aggressive conspecific, or a snake) in a known environment. The next day, the animal is exposed to the same environment and has the possibility through a corridor to enter or not the predator cage (Gross and Canteras, 2012). Passive avoidance can also be implemented by using a two-compartment behavior apparatus, with a shock grid floor, the animal will receive a shock in the preferred compartment. The latency to enter the shock compartment again will be measured (Ambrogi Lorenzini et al., 1999; LeDoux, et al. 2017). The passive avoidance tasks are interesting paradigms, as the acquisition is very rapid and hard to extinct, even with the lack of negative reinforcement. Passive avoidance gives also the possibility to vary the nature of the threat (shock, predator exposure as a snake or a cat).

In our case, we used a novel paradigm previously implanted in our lab in rats (see Viellard et al, 2016). we used an experimental apparatus developed for our experiments of fear conditioning to social and predatory threats as described above. In this case, the animal enters a shock-grid cage where it receives a series of shocks and is exposed to the whole apparatus (safe cage, corridor and grid cage) the next day, where the fear responses are measured.

2) Summary of the structures involved in conditioned active avoidance.

a/ structures involved in signalled active avoidance

As described before, SigA paradigm involves complex mechanisms of conditioning learning and strategy adaptation. According to the Two-factors theory, in early-training phases, active avoidance learning depends on Pavlovian associative processes and lead to increased fear, expressed in terms of freezing. In a second step, avoidance responses are developed depending on instrumental associative processes to ultimately reduce the negative state generated by the CS presentation (Ledoux et al, 2017; Mowrer, 1947). In several avoidance studies both freezing and avoidance are quantified allowing to assess the effect of lesions on both freezing and avoidance behaviors in the same paradigm. So far, the literature emphasizes a strong role of the medial Prefrontal Cortex (mPFC) in

coping strategy selection. It is a nucleus involved in higher processes, regulating a broad range of brain functions related to attention, executive control or working memory (Euston, 2012; Smith 2016). It is also broadly investigated for its role in the regulation of emotional behavior as it is well known that the dysfunction of the mPFC is related to psychiatric conditions such as post-traumatic stress disorder (PTSD) (Shin and Liberzon, 2010). In the case of active avoidance, it is thought that lesions of the mPFC (ACC, IL and PL) disrupt the acquisition but not the expression of goal-directed behaviors pre-training (Gabriel et al, 1991). Lesions of the IL (the ventromedial PFC) region increased freezing expression and disrupted two-way active avoidance learning (Moscarello and LeDoux, 2016). Furthermore, according to Moscarello and colleagues, the expression of passive freezing behavior and active avoidance are inversely correlated and depends on a balance of activity between the IL and the amygdala. Moreover other studies using different active avoidance paradigm show a role of the PL, and the ACC in the acquisition of avoidance learning (Bravo-Olivera et al, 2014). It is important to note that some studies are contradicting these data, saying that neither the PL nor the IL has a role in avoidance learning (Garcia et al, 2006). That is why for the time being, the dorso-ventral axis of the mPFC depending on conditions, doesn't have a clean frontier in terms of role in acquisition and expression of avoidance. One of the many targets of the mPFC is the Ventral Striatum, a particular region of this structure that seems to be involved in active avoidance would be the Nucleus Accumbens (Nac). Even though it has been widely studied in reward and appetitive reinforcement, some studies emphasized its role in acquisition of avoidance learning (Bravo-Olivera et al, 2014) and discrimination of the aversive CS with neutral tones (Oleson et al., 2012). There seems to be a complex implication of the core of the Nucleus acumbens core (NacC) and the shell (NacS) that are respectively, involved in the acquisition and the expression of active avoidance (Moscarello and LeDoux, 2013). However the role of the NacC is still unclear as contradictory studies have been published refuting its role in acquisition (Corbit et al., 2001; Ramirez et al., 2015). Furthermore, the Amygdala is indeed a structure broadly studied for its role in classical fear conditioning and freezing expression (Maren et al., 2001; Herry et al., 2006). Is also a candidate for active avoidance, but working in an

opposite manner as it does for freezing. Indeed amygdala nuclei are reported to participate differentially in avoidance acquisition. First, the LA is shown to be crucial for the acquisition of both freezing and avoidance behaviors (Amaropanth et al., 2002). The BLA and LA but not the CeA impaired the acquisition of Sidman active avoidance behavior in rodents (Lázaro-Muñoz et al., 2010). Even if the LA is an important site for storing the CS-US association, there are probably other circuits regulating that same function as the lesion of the latter impairs early sessions of active avoidance learning, but not the late ones. Moreover, lesioning the CeA nucleus blocks freezing responses and can facilitate avoidance behavior learning in bad performers (Lázaro-Muñoz, et al., 2010). In conclusion the amygdala seems to be crucial in short term avoidance expression but probably relays the information to other systems for long term memory, which could be involving the ventral hippocampus. Indeed a study demonstrated that the ventral hippocampus contributes to the two-way sigA learning (Ang et al. 2015). Another structure that could be part of a putative pathway for avoidance processing, is the periaqueductal gray matter. The first evidence of the involvement of the PAG in mediating defensive behavior was carried out by Bandler and colleagues. The injection of excitatory Amino acid in different parts of the PAG shed light on the different roles of its columns (Keay and Bandler, 2001). The dorsal PAG is a key structure for flight responses and other active behaviors like aggression (Motta et al., 2017). Whereas the ventral columns are inducing more passive behaviors like freezing (Carrive, 1993; Kim et al, 2013). However electrical stimulations of the dorsal PAG of different intensity induce first freezing then flight responses (Vianna et al., 2001). These works point out the dual role of the dorsal PAG on active and passive behaviors, and the complexity of the PAG columns communication. Likewise more recent studies on a communication circuit between the ventrolateral and the dorsolateral PAG showed that the activation of the dlPAG glutamatergic projections to the vlPAG blocks freezing and promotes active defensive behavior expression (Tovote, et al., 2016). The dmPFC and Lateral Hypothalamus are potential candidates to mediate this circuit, as they both projects on the dlPAG (Halladay and Blair, 2015).

To summarize, various structures of the brain have a role in either active avoidance or freezing, but specific studies show a clear role of the mPFC, the amygdalar nuclei and the PAG in monitoring active avoidance system as well as freezing expression.

b/Hypothesis and interests of the study

The literature suggests that, among other structures, the interaction between the Amygdala, the mPFC and the PAG are key structures for driving adapted fear behaviors. Yet it is still unclear if freezing and active avoidance rely on the same, or different circuits. And the structure involved in processing avoidance behavior and the contribution of distinct prefrontal circuits to both freezing and avoidance responses are largely unknown. Our interest is to understand which projection of the dmPFC is a key switch between avoidance and freezing. The role of the amygdalar nuclei, as described above is major in these two behaviors, however they don't seem to have the same dynamic while processing them. That is why our attention focused on the dlPAG, considering the fact that the structures host neural processes implicated in both behaviors. To further investigate the role of dmPFC circuits in encoding passive and active fear coping strategies, in the laboratory of Cyril Herry, I worked in collaboration with Suzana Khoder who had developed a novel behavioral paradigm in which a mouse has the possibility to either passively freeze to an aversive stimulus, or to actively avoid it, depending on the contextual contingencies. Using this behavioral paradigm we investigated whether the same circuits mediate freezing and avoidance behaviors or if distinct neuronal circuits were involved. To address this question, a combination of behavioral, neuronal tracing, immunochemistry, single-unit, patch-clamp recordings and optogenetic techniques were used to study the role of the dmPFC to dlPAG pathway in both active avoidance and freezing acquisition and expression. As Dr. Suzana Khoder published her thesis last year, I will briefly explain in this introduction a part of the conclusions of her thesis, and develop with more details my contribution to the work in the "Result" section of part I.

After validating the behavior paradigm, it was demonstrated that the active avoidance learning paradigm using a two way shuttle box showed variability of learning between the two groups. The good avoiders, who would discriminate the task and learn

to avoid at the tone onset (CS) avoiding the shock, by shuttling to the other compartment, in the open door situation. When the door remained closed during the CS paired to a shock (CS+), the good avoiders also learned to freeze and discriminate with the unpaired CS (CS-). The bad avoiders were unable to learn the task after six days of training, and would freeze more in the closed door situation. However they were able to discriminate between the CS+ and the CS-.

Using in vivo electrophysiological recordings, the results showed that the dmPFC of Good avoiders indicated that most avoidance-inhibited dmPFC PPNs (putative pyramidal neurons) are modulated by both freezing and avoidance, while most avoidance-activated dmPFC PPNs are modulated exclusively by avoidance behavior. Moreover, it has also been demonstrated that changes in firing activity of avoidance-activated dmPFC neurons is not an effect of an increase in locomotion during avoidance and likely reflects associative learning. Furthermore the antidromic stimulations data clearly indicated that the subpopulation of dmPFC PPNs neurons exhibiting an increased activity during avoidance learning (avoidance-activated / freezing non responsive cells) project to the dlPAG.

It was then interesting to take advantage of the fact that a subgroup of animals could not learn the avoidance task. In this view the PL to dlPAG pathway was activated using optogenetic tools. The data pointed out that optogenetic stimulation of dmPFC-dl/lPAG projections progressively promotes learning of avoidance behavior. Once again it has also been proved that the 10Hz optogenetic stimulation of the pathway is not a locomotor effect. Thus, supporting the electrophysiological results, the activation of dmPFC neurons projecting to the dl/lPAG did not affect conditioned freezing behavior.

To reinforce these data, it was also demonstrated using in vitro whole cell recordings by measuring the AMPA/NMDA receptor ratio, that the switch of Bad avoiders into Good avoiders upon the optogenetic stimulation of the dmPFC dl/lPAG pathway is associated with the development of synaptic potentiation at dmPFC inputs onto dl/lPAG cells. (see "Role of the prefrontal-brainstem pathway in mediating avoidance behavior", Suzana Khoder, thesis 2018)

In order to give a stronger insight on these differences between the two groups, my contribution to the work was to investigate the activation pattern of the mPFC and the PAG, in good and bad avoiders. Also, it is still unclear, with the optogenetic activation of the pathway only, to understand if the cells projecting from the dmPFC to the dlPAG are involved in the expression of avoidance and/or its acquisition. These questions will then be assessed using Fos immunochemistry analysis, inhibitory optogenetic strategies, and other behavioral tests.

3) Summary of the structures involved in conditioned passive avoidance.

a/ structures involved in passive avoidance

Passive avoidance is a non instrumental form of avoidance and has been studied through lesion studies, using inhibitory avoidance tasks. For instance, the literature indicates that the mPFC is a potential structure to mediate passive avoidance. Indeed, studies of the mPFC demonstrate that lesions of the PL in rats in the step-through passive avoidance paradigm impaired fear memory whereas a stimulation of the region improves it (Canto de Souza et al., 2016). The ventral hippocampus and lateral septum also have an important role in the encoding of association of contextual cues (Gross and Canteras, 2012). In fact, the septo-hippocampal system has been proposed to play a pivotal role in anxiety in response to conflicted situations, by interrupting ongoing behavior and increasing the level of arousal and attention to enhance gathering information (Gray and McNaughton, 2000). It is also known that anxiogenic-like state is provoked by selective stimulation of BLA to ventral hippocampus projections. And the stimulation of the amygdala has been shown to disrupt inhibitory passive avoidance (Gold, et al., 1973). Notably, the striatum has been demonstrated to play a role, but as opposed to active avoidance, fear retrieval following conditioning is disrupted by nucleus accumbens shell, but not core, region inactivation (Piantadosi, et al., 2018). It is important to note that most of the data on passive avoidance were collected using step-through or step-down inhibitory avoidance paradigms. The difference with the paradigm used in this study is the proximity with the threatening location. The presence of a corridor imposes a

distance between the safe cage and the conditioning cage, and this situation may need the recruitment of particular neural circuits for the encoding and retrieval of fearful contextual cues. So far, according to a fos study in rats using this experimental apparatus on shock based passive avoidance, the circuit recruited during fear retrieval seems to involve a specific septo/hippocampal-hypothalamic-brainstem pathway, namely the ventral subiculum, lateral septum, the juxtadorsomedial part of the lateral hypothalamus (LHAjd), the dorsal premammillary nucleus (PMD) and the dorsal and lateral parts of the PAG (dl/lPAG) (Viellard et al., 2016). Notably, the PMD occupies a pivotal role in this circuit, and its hodological relationships will be discussed below.

b/Hypothesis and interests of the study

According to recent work of our lab, and the literature on innate fear learning, we are understanding that shock-based contextual passive avoidance is mediated in part by a septo/hippocampal-hypothalamic-brainstem pathway. We are hypothesising that a key structure of this pathway is the PMD. The PMD is largely influenced by septo-hippocampal processing (Comoli et al., 2000) and, on the efferent side, sends projections to both the AMv (likely to be involved in encoding fear memory) and the dorsal PAG, which is known to participate in the expression of active and passive defensive behaviors. After validating the activation of this septo/hippocampal-hypothalamic-brainstem pathway in mouse using a Fos immunochemistry analysis, we will focus on the PMD and its projection sites using a combination of behavioral, pharmacogenetic and optogenetic approaches, to evaluate their roles on the expression of inhibitory avoidance and fear memory reconsolidation process.

4) Anatomy and connectivity of our structures of interest

This next paragraph will give a rapid insight on the anatomy of our two structures of interest: the dmPFC and the PMD.

a/ The dorso-medial Prefrontal Cortex

The rodent mPFC can be divided into four distinct areas which are, descending from the most dorsal region, the medial precentral cortex (PrCm), the Anterior Cingulate Cortex (ACC) (dorsal and ventral part), the Prelimbic Cortex and the Infralimbic Cortex. Our interest will focus on the dorsal part of the medial cortex, which are the PL and the ACC. The ACC areas regulates various motor behavior, whereas the PL will regulate emotional, mnemonic, and cognitive processes (Heidbreder and Groenewegen, 2003). The cortex has a paralleled laminar organization divided in 6 layers that are called from the most superficial to the deepest: the molecular layer, which is poorly dense in neurons; the external granular layer; the external pyramidal layer; The layer 4, or granular layer, (this layer is not present in rodents); the internal pyramidal layer, that is composed of sparse and large Pyramidal neurons (PNs) vertically oriented; and lastly the polymorph layer, that contains various neuronal types without specific organization. These six layers have their own organization in terms of connectivity. For instance, layers 2/3 support the cortico-cortical connections, layers 1 and 4 receive thalamic inputs and layers 5 and 6 are respectively the main sources of thalamic and subthalamic projections (Harris and Shepherd, 2015).

The cortex is composed of two main classes of neurons: the glutamatergic pyramidal neurons (PNs) and the GABAergic interneurons (INs) that represent respectively 80% and 20% of the cortical neurons. PNs used glutamate as a neurotransmitter and are located in all six cortical layers, except layer 1. As opposed to PNs, the vast majority of INs do not leave the cortex and are restricted to a local environment (Spruston, 2008). That is why our projection studies will focus on PNs. Finally, INs can be characterized at neurochemical level, indeed a numerous variety of peptides in encountered in interneurons, that give them their neuronal subtypes (i.e. PV, CR, CB, SST, VIP, CCK, NYP). Because of their morphological, electrophysiological and molecular diversity, INs are believed to differentially sculpt cortical activity. (Hubel and Wiesel, 1962).

Inputs: The dmPFC, our structure of interest, receives its major inputs from the medial segment of the thalamus (MD), it also projects back through a descending pathway to the MD (Groenewegen, 1988). The whole PFC, including the PL receives also massive inputs from the BLA. The paralimbic cortex sends reciprocal projections back to the PL. Another important input is coming from the vHPC (CA1 region and subiculum) and terminates in all layers of the PL, with sparse inputs from the dorsal hippocampus.

outputs: The ACC and PL project mostly to the BA, as opposed to the IL for example, that will send projections to the CeA and LA (Hoover and Vertes, 2007). The mPFC shares reciprocal connectivity with the ventral tegmental area (VTA), the basal ganglia (Groenewegen et al., 1988), and the dorsal and lateral regions of the PAG (Gabbott et al., 2005). It also projects to the hypothalamus, like the PMD (Comoli et al., 2000). The PL also project internally to the ventral ACC and the IL region sending outputs preferentially to the PrCm and dorsal ACC (Hoover and Vertes, 2007).

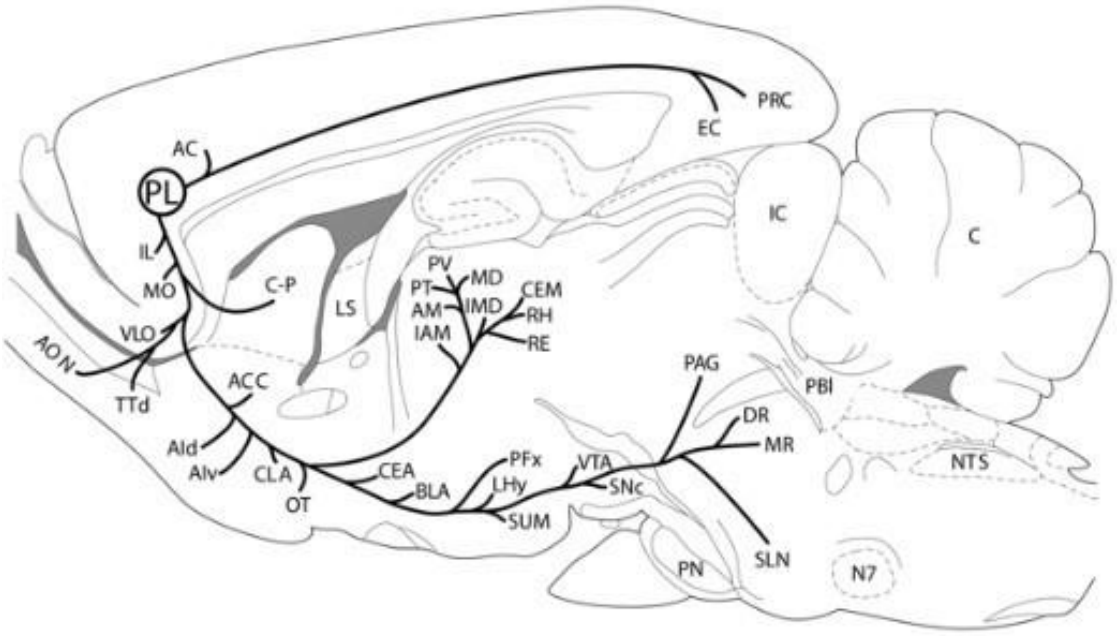

Figure 1 Schematic sagittal sections summarizing the main efferents projections of the PL in rats Sections are modified from the rat atlas of Paxinos and Franklin (Paxinos and Franklin, 2008)

Abbreviations: AA, anterior area of amygdala; AHN, anterior nucleus of hypothalamus; AI,d,v, agranular insular cortex, dorsal, ventral divisions; AM, anteromedial nucleus of thalamus; AON, anterior olfactory nucleus; BMA, basomedial nucleus of amygdala; C, cerebellum; CEM, central medial nucleus of thalamus; CLA, claustrum; COA, cortical nucleus of amygdala; C-P, caudate/putamen; DBh, nucleus of the diagonal band, horizontal limb; DMH, dorsomedial nucleus of hypothalamus; DR, dorsal raphe nucleus; EN, endopiriform nucleus; IAM, interanteromedial nucleus of thalamus; IC, inferior colliculus; IMD, intermediodorsal nucleus of thalamus; IP, interpeduncular nucleus; LHy, lateral hypothalamic area; LPO, lateral preoptic area; LS, lateral septal nucleus; MEA, medial nucleus of amygdala; MO, medial orbital cortex; MPO, medial preoptic area; MR, median raphe nucleus; N7, facial nucleus; OT, olfactory tubercle; PBm,l, parabrachial nucleus, medial and lateral divisions; PFx, perifornical region of hypothalamus; PN, nucleus of pons; PRC, Reuniens nucleus; RE, perirhinal cortex; RH, rhomboid nucleus of thalamus; SI, substantia innominata; SLN, supralemniscal nuc leus (B9); SUM, supramammillary nucleus; TTd, taenia tecta, dorsal part; VLO, ventral lateral orbital cortex; VO, ventral orbital cortex. Reprinted from Vertes (2004).

b/ The dorsal Premammillary nucleus of the Hypothalamus

The PMD is a small dense structure of the posterior ventral hypothalamus with anatomical and neuronal properties that are poorly understood. It is part of the mammilar complex but has unique projections and functions compare to the other mammillar nuclei (Canteras and Swanson, 1992). It is known to be a structure mostly glutamatergic.

inputs: The PMD receives a dense input from the ventral tegmental nucleus , and unlike other mammillary nuclei, the PMD does not receive a direct input from subicular regions of the hippocampal formation but instead it receives a massive input from the anterior hypothalamic nucleus and the juxtadorsomedial part of the lateral hypothalamic area (LHAjd) (Comoli et al., 2000; Hahn and Swanson, 2012).The anterior hypothalamic nucleus integrates and transmits (either directly or indirectly) information from the prefrontal cortex, amygdala, hippocampus, and septal region, whereas the LHAjd receives massive inputs from the subiculum and lateral septum (Comoli et al., 2000; Hahn and Swanson, 2012). Moreover, more sparse inputs to the PMD arise from the ventromedial hypothalamus (VHM)and prelimbic cortex PL (Comoli et al, 2000; Canteras and Swanson, 1992)

Outputs: ascending branch of the PMD projection ends massively in the ventral part of the anteromedial nucleus of the thalamus (AMv) and anterior hypothalamic nucleus (AHN); this branch also provides moderate inputs to rostral parts of the zona incerta, the nucleus reuniens, and to perifornical areas of the lateral hypothalamic area. The descending branch of the PMD projection courses to and through the posterior hypothalamic nucleus and end densely in the dorsolateral part, but also the medial and lateral parts of the periaqueductal gray; as well as in the deep and intermediate gray layers of the superior colliculus, and caudal parts of the midbrain reticular formation (including the cuneiform nucleus) (Canteras and Swanson, 1992).

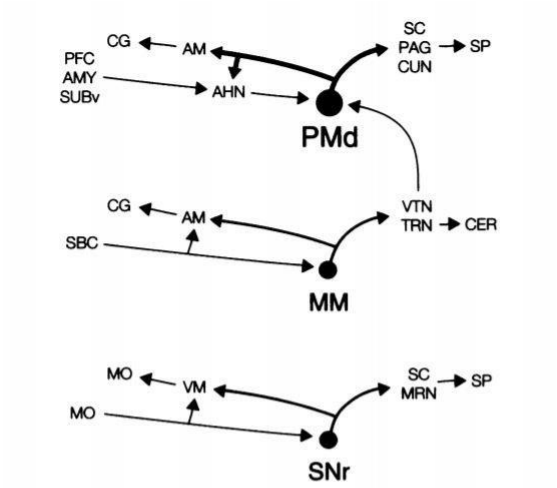

FIG. 4. Major input/output relations of the PMd, medial mammillary nucleus (MM), and reticular part of substantia nigra (SNr). Hypothetical pathway from the periaqueductal gray (PAG) to the spinal cord (SP) may be direct or indirect. AHN, anterior hypothalamic nucleus (n.); AM, anteromedial n. thalamus; AMY, amygdala; CER, cerebellum; CG, cingulate gyrus; CUN, cuneiform n.; MO, motor cortex; MRN, mesencephalic reticular n.; PAG, periaqueductal gray; PFC, prefrontal cortex; SBC, subicular complex; SC, superior colliculus; SP, spinal cord; SUBv, ventral subiculum; TRN, tegmental reticular n.; VM, ventral medial n. thalamus; VTN, ventral tegmental n.

Figure 2 . Scheme of the inputs and outputs related to the PMD (Canteras and Swanson, 1992)

Discussion

First Part

A novel behavioral paradigm to study avoidance and freezing: In the first part of the project we focused our attention on the projection of the dmPFC to the dlPAG, and its role in avoidance and freezing acquisition and expression. To that extent we developed a novel behavioral paradigm of active avoidance during which a single CS was associated with two conditioned behavioral outcomes (freezing versus avoidance) depending on the state of the door in between compartments. Freezing behavior was evident to acquire for all the mice tested in our paradigm. All mice froze significantly more to the CS+ (between 40% to 60 % in DC condition) compared to the CS- and, discriminated between the two CSs. Interestingly, the freezing level evoked by the CS- was relatively high in all mice. The fact that mice cannot predict whether the door will open or stay close increases the attentional processes and promotes immobility. It could be an explanation of their high freezing level during CS-. A second potential explanation could be linked to the random trial presentation. Even though random presentation of different trial types makes the learning more complex, it also potentially enhances attentional processes and prevents the development of habitual avoidance learning (Dickinson, 1985; Wood and Neal, 2007). Our objective being to study goal-directed avoidance learning and not habitual avoidance we opted for presenting the trials in an intermingled manner.

Interestingly, the second behavioral outcome (avoidance) was not learned by all mice. Indeed, we categorized mice based on (i) avoidance scores and (ii) discrimination between CS- and CS+ trials. This categorization led to Bad avoiders (mice that did not learn to avoid), Good avoiders (mice that learned discriminative avoidance) and generalizers (Good avoiders that learned to shuttle/avoid to the other compartment each time the door got opened regardless of the CS). In terms of freezing, Bad avoiders, Good avoiders and generalizers also differ at two levels even though all three groups discriminate between CS- and CS+. During DC condition, Bad avoiders present the highest freezing levels to CS+ (mean~55-60%) followed by discriminators (mean~45-50%) and generalizers which exhibited very low freezing levels to CS+ presentations (mean~35-

40%). Therefore, it seems that the DC condition allows to categorize animals in terms of freezing levels. During DO trials, at door opening Bad avoiders continue to freeze at high levels post-DO whereas Good avoiders and generalizers present a drop in their freezing levels since they switch to an active defensive strategy.

This heterogeneity in acquiring active defensive strategies have been already reported in active avoidance studies (Galatzer-Levy, et al., 2014) and is of a relative importance from a clinical point of view because it transduces the heterogeneity of response of humans facing traumatic events. Regarding the proportions of the different behavioral profiles, both the original paradigm and the simplified adapted paradigm (only DO condition) resulted in ~35% of Good avoiders discriminators which acquired discriminative avoidance behavior. For connected animals (optic fibers, electrodes cables), around 45 to 55% of the population were classified as Bad avoiders and the rest as Generalizers. In all the experiments, generalizers were not further considered.

Regarding the FST, the results showed that there was no difference between the two groups of animals. This test was important in this study to measure the impact of repetitive shocks on the animals. As the FST induces learned helplessness (Yankelevitch-Yahav, et al., 2015), it is important to note that the bad avoiders did not change their strategy, in this test, even though failing to learn the avoidance task. However it doesn't give us insight on the background of the animals, and why such a great number of them cannot learn the task. Even though the two way avoidance shuttle box paradigm doesn't impact the emotional state of the bad avoiders, extreme behaviors (the non avoider and the generalizer) are however reflected in the FST, as the non-avoider almost drowned, and the generalizer did not stop swimming. We can then advance, that abnormal stress level in a small portion of the animals impacted their performance of the two-way SigA paradigm. Some studies also suggest that the FST reveal a certain heterogeneity in immobility levels after chronic and acute stress experiments (Suvrathan et al., 2010). To validate this hypothesis, an interesting experiment would be to administer an anxiolytic drug before each SigA training day in order to see if the proportion of good and bad avoiders will be different. It can then also be interesting to study how afferent

hypothalamic projections, like the PVH, known to be involved in stress (Xu et al., 2019), would indirectly impact the dmPFC to dlPAG pathway.

The active avoidance learning preferentially activates the dmPFC and the cdlPAG: The immediate-early gene c-fos study we performed revealed a clear significant upregulation of c-fos in Good avoiders as compared to Bad avoiders and controls in the caudal dmPFC (ACC, PL). Our results are in concordance with several studies in rodents using a platform-mediated avoidance paradigm (Bravo-Rivera, et al., 2015) or lever-press avoidance paradigm (Beck, et al., 2014) demonstrating that PL drives avoidance behavior acquisition/expression. Our results are also consistent with clinical results indicating that in healthy humans avoidance is linked to an increased reactivity of the anterior ACC and the dmPFC (Schlund, et al., 2015). Based on our fos results, we also identified a structure considered to regulate the defensive output behavioral responses, namely the PAG and more specifically the dlPAG. Even though the dlPAG show a clear upregulation in the animals performing the avoidance task, this structure did not show a significative difference between the good and the bad avoiders. It is however important to note that no direct correlation between the dlPAG Fos upregulation and freezing was found (data not shown). As mentioned before, the dlPAG is involved in both passive and active defensive responses (Viana et al., 2001). Knowing that can explain the fact that the dlPAG could have been recruited by the dmPFC in the case of the good avoiders performing avoidance; and by other direct inputs as the hypothalamus, or indirect inputs as the Amydgala via the vlPAG, in the case of bad avoiders with a higher freezing level (Halladay and Blair, 2015, Rozeske, et al., 2018, Tovote, et al., 2016).

The caudal dmPFC promotes active avoidance : Using the conclusions brought by our in vivo electrophysiological, antidromic and optogenetic data, we can strongly suggest that avoidance behavior is driven by an activation of a subpopulation of dmPFC PNs. (see Khoder, 2018) which opposes the results of a recent paper (Diehl, et al., 2018) suggesting that avoidance is rather associated with an inhibition of dmPFC activity. We think that those discrepancies are linked to the differences in the rostro-caudal axis of manipulation/recordings at the dmPFC level. Indeed our recordings in the dmPFC and

optogenetic manipulations are made in the caudal dmPFC (A.P. < +2.1) whereas in the platform-mediated paradigm (Diehl, et al., 2018) the results concern the rostral dmPFC (A.P. > 2.1). Furthermore, a pilot study from our lab tends to show that inhibiting the rostral dmPFC to dlPAG pathway promotes avoidance learning, whereas the caudal pathway abolishes it. The opposing roles in avoidance learning played by the rostral dmPFC and caudal dmPFC rise an important question, being to determine which structure is critically involved in the selection of the behavioral response during avoidance. Does the selection of avoidance behavior depend on the rostral vs caudal dmPFC local connectivity or is the selection made at downstream structures like the dl/lPAG? It also reopens the question about where the behavioral switch between freezing and avoidance is made. We demonstrated here that the caudal PL was specifically involved in avoidance learning, and not freezing. However, as our recordings in the cdmPFC infer, there are cells activated in both freezing and avoidance (Khoder, 2018). rostral dmPFC to dlPAG is yet to be studied to understand its role in this behavioral switch. Additional experiments will be required to specifically address this question.

The dmPFC to dlPAG pathway is necessary for promoting avoidance behavior but not freezing: The modulation of the dmPFC to dlPAG pathway was made in two steps. Firstly we activated the pathway in the bad avoiders to see the evolution of their performance in the two way SigA paradigm. Interestingly, the two days of post training stimulation made the bad avoiders improve their avoidance performances, and the animals discriminate better the CS+ and the CS-. However the freezing level in DC condition was unchanged (see Khoder, 2018). The fact that the animals kept improving after the stimulation days, showed us that the stimulation of the pathway couldn't promote avoidance expression only. This hypothesis was clarified with the inhibition of the pathway in the good avoiders. In fact early training inhibition but not post training inhibition impaired their capacity to acquire avoidance. These results validated the fact that the dmPFC pathway is sufficient and necessary to promote avoidance behavior. However its role in freezing is not present. These results refute the fact that freezing and avoidance are mediated by the same caudal dmPFC to dlPAG pathway, and go along with other works emphasizing the involvement of the dmPFC in platform-mediated avoidance

but not freezing behavior (Bravo-Rivera, et al., 2014; Diehl, et al, 2018). However as opposed to these works, our electrophysiological data showed that a proportion cdmPFC neurons encode for freezing only. It is then more likely that freezing and avoidance are driven in the dmPFC by independent subsets of neuron populations. A possible candidate mediating freezing expression alone would be the dmPFC to BLA pathway, as it is proposed in the literature (Courtin, et al., 2014).

Second Part

Behaviors involved in the passive avoidance paradigm: In the second part, using the paradigm based on our previous work on rats, we were able to reproduce the results in mice (Viellard et al. 2016). The two groups of animals presented clear behavioral differences in terms of contextual fear responses. Following the day of the conditioning, the group kept enclosed in the in the shock chamber, spent close to 25% of the time frozen, and 51% of the time immobile in a crouched back posture, sniffing the environment (crouch sniff; Blanchard and Blanchard, 1969). In contrast, animals placed in the home cage with open access to the conditioning chamber presented only a minimal amount of freezing (1%), and spent most of the time of the test risk assessing the environment with either crouch sniff (61%) or doing stretch postures (21%). This group of animals did not enter the conditioning chamber and, in addition to the fear responses, also actively explore the home box and corridor close to 17% of the time. The groups that did not receive shocks actively explored their environment fearlessly in both conditions. As reported before, the different conditions in which the animal recalls a threatful environment, affects the responses of the animal (Viellard et al., 2016). The present findings of this shock-based passive avoidance paradigm can be compared with previous studies from our lab using the same apparatus and experimental design for either cat exposure or social defeat. As we have just found for footshock conditioning, animals conditioned with either predator threat or social defeat, presented a similar form of contextual fear responses (i.e., risk assessment). And when placed in the home cage with access to the compartment associated with either predator threat or social defeat, they largely avoided this chamber. The amplitude of the fear response is difficult to compare as the impact of the different threats (i.e., footshock, aggressive conspecific and

predator) on the animal is not measurable. However, compared to other shock-based passive avoidance paradigms, this one leaves a stronger conditioning as the animal entirely avoid the conditioning chamber. In our case, on the conditioning day, the animal received a series of shocks enclosed in the conditioning chamber, whereas in the step-down inhibitory avoidance another form of shock-based passive avoidance paradigm, the animal has the possibility to escape after the first shock (Ambrogi Lorenzini, et al., 1999). Furthermore the long term pre-exposure habituation (three days) is known to influence the conditioning process, as it has been shown that context pre-exposure facilitates and strengthen the learning of context-shock association (Fanselow , 1980; Rudy 2009). One could argue that a strong conditioning as the one in our paradigm could lead to generalization. However, the results in the open field showed the behavioral ratio between fearful and fearless behaviors does not change after the conditioning day, whereas this ratio greatly increases in the conditioning apparatus. Thus suggesting that the animals differentiate the aversive and neutral contexts. The experiment was also set using mild shocks of 0.6mA, which are unlikely to create generalization (Baldi et al., 2004). Compared to animals tested enclosed in conditioning cage, the present paradigm (using a shock as a controllable threat) yields the expression of a larger range of risk assessment behaviors, which are good candidates for modeling anxiety behaviors in humans (Blanchard, 2019).

Septo-hippocampal–hypothalamic-brainstem circuit putatively involved in inhibitory avoidance: comparison to other threats and conditions: The present results are also in line with our previous results, in rats (Viellard et al., 2016), showing that the fear conditioned animals, which were able to avoid the conditioning chamber, presented increased Fos expression in a circuit formed by the subiculum, the lateral septum, the juxtadorsomedial part of lateral hypothalamic area (LHAjd), the dorsal premammillary nucleus and the lateral and dorsal parts of the periaqueductal gray. Anatomical and functional data suggest that this septo/hippocampal-hypothalamic-brainstem circuit should be putatively involved in mediating contextual avoidance. Interestingly, social defeat to an aggressive conspecific, exposure to a snake predator and restraint stress also

up-regulate Fos expression in this same circuit. Notably, in response to all these threats, animals displayed a significant increase in Fos expression in the juxtadorsomedial region of the lateral hypothalamic area (LHAjd) (Motta and Canteras, 2015; Tessari et al., 2019). The LHAjd conveys information to the dorsal premammillary nucleus from the septo-hippocampal system (Hahn and Swanson, 2012). The septo-hippocampal system has been proposed to play a pivotal role in anxiety in response to conflict situations, by interrupting ongoing behavior and increasing the level of arousal and attention to enhance gathering information (Gray and McNaughton, 2000). In fact, the hippocampus may work as a context analyzer providing a spatial mapping of the environment derived from two sets of information: one based on the external environment and the other based on self-motion (Burgess et al., 2002). Of relevance to the present study, the hippocampus contains a special kind of cell, the boundary vector cell (BVC), which codes for environmental boundaries (irrespective of their sensory nature (Stewart et al., 2013)). Interestingly, the distribution of the BVCs and the cells that project to the LHAjd seem to overlap, at least partially, in the subiculum (Hahn and Swanson, 2012). The concept of an environmental boundary is somewhat abstract and represents an effective obstacle to locomotion that does not necessarily involve physical prevention of movement (Stewart et al., 2013). Considering the evidences, all these forms of threats (i.e., physical constraint, exposure to an aggressive conspecific or a snake predator, and the avoidance of a threatening chamber) set clear environmental boundaries, constraining the animals either physically (by the restraining apparatus) or behaviorally (conspecific aggressor, snake predator, and the threatening chamber). Therefore, it would be interesting to investigate whether the avoidance of the threatening chamber would work as an environmental boundary signaled by BVC cells. As previously mentioned, on the efferent side, the LHAjd projects densely to the dorsal premammillary nucleus (PMD), in addition to the dorsomedial and lateral parts of the periaqueductal gray (PAGdm,l) (Hahn and Swanson, 2012), all of which have been shown to present a significant Fos increase in response to passive avoidance, as well as to a social aggressor and snake threat (Motta et al., 2009; Faturi et al., 2014; Tessari et al., 2019). The present results gives further support to the idea that there are interesting commonalities among restraint stress,

social defeat, snake threat and passive contextual avoidance, suggesting a septo-hippocampal–hypothalamic-brainstem path likely to respond to the environmental boundary restriction that may act as common stressor component for all these types of stress.

PMD influences both inhibitory avoidance and memory re-consolidation: The PMD has a pivotal role in the septo/hippocampal-hypothalamic-brainstem circuit putatively involved in mediating contextual avoidance. On the afferent side, the PMD integrates hippocampal information likely related to signaling environmental boundaries, and on the efferent side, the nucleus projects to the periaqueductal gray, which is critically involved in the expression of avoidance responses (Motta et al., 2017). The present results indicate that pharmacogenetic inhibition of the PMD resulted in a general decrease in risk assessment behaviors. Thus, the CNO-injected animals expressing hM4D receptor in the PMD spent around 150 seconds risk assessing the shock-related context of the context exposure, in comparison to close to 300 seconds for the control group. Moreover, the group in which the PMD was inhibited spend about 130 seconds exploring the conditioning cage whereas the control group did not enter this cage. Notably, in the CCK CRE line used in this experiment, apart from the PMD, the expression of the hM4D receptor spread to a certain degree over the mammillary bodies, which also contain CCK cell bodies. However, CNO-injected animals containing the hM4D receptor only in the mammillary bodies did not reduce risk assessment and did not enter the shock-related chamber during the day after the conditioning. Moreover, our Fos analysis showed no involvement of the mammillary bodies in passive avoidance. In line with the present results, pharmacological inactivation of the PMD, but not of the nearby mammillary nuclei, was able to significantly reduce the contextual conditioned responses to predatory threats (Cezario et al., 2008). As in the present case, in this experiment, animals were tested in a similar apparatus with a home cage linked to a corridor and the threatening chamber, and muscimol injection in the PMD, on the day after cat exposure, drastically reduced risk assessment responses and the animal entered the threatening chamber (Cezario et al., 2008).

On the day following PMD inhibition, we found a decrease in the inhibitory avoidance in the animals re-exposed to the threatening context. Thus, suggesting that

PMD inhibition influenced memory processes related to fear re-consolidation during exposure to the threatening environment. Accordingly, the group of animals expressing hM4D in the PMD that received CNO during the first day of exposure to the shock-related environment, when re-tested the following day in the same context, presented decreased risk assessment responses and spent significantly higher amount of time in the conditioning compartment. In line with the present results, pharmacological blockade of either beta-adrenoceptor or NMDA receptor in the PMD, but not in the adjacent mammillary bodies, immediately before the conditioning session, reduced the defensive response to the cat odor and also, 24 hours later, to the cat-odor related environment (Canteras et al., 2008; Do Monte et al., 2008). The PMD's role in fear memory may be viewed as either an impairment in fear memory processing or the result of decreased emotional component of the aversive event during the learning stage. In favor of the view that the decrease of emotional component during the learning stage does not necessarily influence fear memory, (De Andrade Rufino et al., 2019) found that ventral periaqueductal gray lesions resulting in clear decrease of innate defensive responses to a predator did not affect anti-predatory contextual fear learning.

Overall, our results indicate that the PMD influences both the expression of inhibitory avoidance and the memory re-consolidation processes during exposure to the shock-related context.

How the PMD's targets influence passive avoidance and fear memory re-consolidation : The functional role of the PMD appears to depend on its branched pathway to the periaqueductal gray (PAG) and the ventral part of the anteromedial thalamic nucleus (AMv) (Canteras and Swanson, 1992). Therefore, we examined how the PMD projections to the PAG and AMv influences defensive responses during the contextual avoidance and the memory re-consolidation process during exposure to the shock-related context. To this end, we induced halorodopsin expression in the PMD cells, and silenced the PMD's terminals in either the PAG or the AMv. At first, we were expecting that silencing the projections to the PAG would influence passive avoidance during exposure to the shock-related context, whereas inactivation of the projections to the AMv would disrupt the memory re-consolidation process. However, inactivation of PMD terminals in the PAG or

in the AMv had similar effects. Thus, optogenetic inhibition of PMD projection to the PAG or to the AMv during exposure to the shock-related context resulted in decreased risk assessment responses and increase the time spent in the conditioning chamber. Moreover, compared to the control group, animals that received optogenetic inhibition of PMD's terminals in the PAG or in the AMv, when re-tested 24 hours late in the same context, presented a reduction in risk assessment responses and spent significantly higher amount of time in the conditioning chamber. Therefore, silencing the PMD's projections to the PAG or the AMv interfere with both the expression of defensive responses during contextual avoidance and the memory reconsolidation process. At this point, we need to understand how the PMD's targets could influence both the expression of defensive responses during the inhibitory avoidance and the memory re-consolidation processing.

Previous studies have shown that pharmacological inactivation of the AMv disrupts the acquisition of contextual memory to predatory threats (De lima et al, 2017). The AMv role on memory processing seems to depend on its projection to a cortical network (formed by the prelimbic, anterior cingulate, visual associative and ventral retrosplenial areas), which influences fear memory and has access to key elements involved in memory processing, such as the basolateral amygdala and the hippocampal formation (De Lima et al., 2019).

In the PAG, particularly its dorsal part has been shown to support fear learning. Of relevance, the dorsal PAG seems critical for the acquisition of contextual fear memory to predator threats (Souza and Carobrez, 2016; De Andrade Rufino et al., 2019). Moreover, several studies using classical fear conditioning to sound-, light- or odor-conditioned stimuli (CS) have shown that electrical, chemical or optogenetic stimulation of the dorsal PAG may work as a useful US to support associative learning (Deng et al., 2016; Di Scala et al., 1987; Di Scala and Sander, 1989; Kincheski et al., 2012; Kim et al., 2013). The dorsal PAG provides a number of parallel thalamic paths likely to influence fear learning. Thus, the dorsal PAG provides direct inputs to the nucleus reuniens, the central lateral nucleus, the lateral dorsal nucleus, the supragenicular nucleus, and the parvicellular subparafascicular nucleus (Kincheski et al., 2012). The nucleus reuniens

represents the main thalamic source of projections to the hippocampal formation (Vertes et al., 2006); the central lateral nucleus and the lateral dorsal nucleus project to cortical areas involved in the cortical circuit mentioned above that influences fear learning (i.e., the anterior cingulate and retrosplenial areas) (Furlong et al., 2010, van Groen and Wyss, 1992); and the suprageniculate and the parvicellular subparafascicular nuclei project densely to the lateral amygdalar nucleus (Linke et al., 2000). However, further studies are needed to address how these dorsal PAG-thalamic pathways may influence fear learning. During exposure to environments previously associated with a threat, such as a predator, an aggressive conspecific or, as in the present case, a footshock, the threat is more ambiguous and evokes risk assessment responses, including a careful scanning of the environment in the crouched position (crouch sniffing) and attempts to approach the threatening stimulus by stretching the body (stretch postures) (Ribeiro-Barbosa et al., 2005; Faturi et al, 2014; Viellard et al., 2016). Previous studies using cytotoxic lesions and pharmacological inactivation have shown that the dorsal PAG appears to exert critical control on risk assessment responses (Faturi et al., 2014; Pobbe et al., 2011). In agreement with this idea, the present results showed that optogenetic inhibition of the PMD´s projection to the PAG, which is putatively a glutamatergic projection, decreased risk assessment response during exposure to the shock-related context. Risk assessment responses are very complex, and it is not clear how the dorsal PAG influences these responses. Nevertheless, ascending dorsal PAG projections to prosencephalic targets have been proposed to influence risk assessment behaviors (Motta et al., 2017).

One of our must puzzling results was the drastic reduction of risk assessment in response to the optogenetic inhibition of the PMD projection to the AMv in animals exposed to the shock-related context. Recent results from our lab indicate that the AMv-related cortical network may influence the expression of contextual fear responses. In this way, we have found that optogenetic inhibition of anterior cingulate projection to the dorsal PAG significantly reduced risk assessment responses during exposure to context previously related to a predator. Therefore, the PMD would influence the expression of inhibitory avoidance during exposure to shock-related context through its

direct projection to the PAG and the through the projection to the AMv, which may ultimately impact on the anterior cingulate area – dorsal PAG pathway.

To help understand better the nature and the specific role of the cells projecting to the AMV and the dlPAG, further experiments will be done using electrophysiogical recordings of PMD cells and try to correlate their firing rate with specific behavioral responses. Concerning the afferent pathway of the PMD, it has been previously noted how important the hippocampus is in passive avoidance and fear learning, and future studies will aim to investigate whether the avoidance of the threatening chamber would work as an environmental boundary perhaps signaled by BVC cells.

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