JULIETTE MARGUERITE ALIX VIELLARD

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

Tese em cotutela apresentada ao programa de Pos Graduação em Biologia de Sistemas do Instituto de Ciências Biomédicas da Universidade de São Paulo, e de Pos Graduação das Ciencias da vida e da Saude da Universidade de Bordeaux para obtenção do título de Doutor em Ciências.

São Paulo
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Orientador da Universidade de São Paulo: Prof. Dr. Newton Sabino Canteras
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Certificamos que o projeto intitulado "***BASES NEURAIS DO MEDO E AGRESSÃO***", registrado sob o protocolo nº ***23/2017***, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de *Pesquisa Científica*, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle e Experimentação Animal (CONCEA). Ante esta conformidade, o referido projeto foi avaliado e aprovado em ***21/03/2017*** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Instituto de Ciências Biomédicas da Universidade de São Paulo (CEUA-ICB/USP), outorgando esta licença de uso de animais com validade de **4 ano(s)** a partir da data de aprovação.

- Investigador Principal: **Dr.(a.)** ***Newton Sabino Canteras***
- Departamento: *Anatomia*
- Membros da Equipe: *Miguel Antonio Xavier de Lima (Credenciado PG), Ignacio Marin Blasco (Credenciado PG), Juliette Marguerite Alix Viellard (Pós-graduando), Miguel José Ranguel Junior (Pós-graduando), Amanda Ribeiro de Oliveira (Especialista de laboratório)*

Ao final do período outorgado por esta licença, o pesquisador responsável deverá encaminhar a esta comissão, até o último dia de validade da atual proposta, *relatório final* de acordo com a Resolução Normativa CONCEA nº 30/2016 - Diretriz Brasileira para o Cuidado e a Utilização de Animais em Atividades de Ensino ou de Pesquisa Científica (DBCA), conforme modelo constante no endereço eletrônico www.icb.usp.br/ceua. Havendo interesse na renovação do projeto, a solicitação deverá ser protocolada pela Secretaria da CEUA-ICB/USP até o último dia de validade da atual proposta. Após esta data uma nova proposta deverá ser encaminhada.

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We hereby certify that the project entitled "***BASIS OF FEAR AND AGGRESSION***", protocol nº ***23/2017***, which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human), for *Scientific Research Purposes*, is in accordance with the provisions of the Law nº 11.794 passed on October 8th, 2008, Decree nº 6899 passed on July 15th, 2009, and the rules issued by the National Council for Control and Animal Experimentation (CONCEA). According to this legislation, the project was evaluated and approved on ***3/21/2017*** by the ETHICS COMMITTEE ON ANIMAL USE, Institute of Biomedical Sciences, University of Sao Paulo (CEUA-ICB/USP), and the license for animal use is valid for **4 year(s)** from the date of approval.

- Principal Investigator: **Dr.(a.)** ***Newton Sabino Canteras***
- Team members: *Miguel Antonio Xavier de Lima (Graduate Supervisor), Ignacio Marin Blasco (Graduate Supervisor), Juliette Marguerite Alix Viellard (Graduate Student), Miguel José Ranguel Junior (Graduate Student), Amanda Ribeiro de Oliveira (Laboratory Technician).*

At the end of the period granted by this license, the Principal Investigator must submit a final report of the project to this committee, according to the Rule nº 30 and the Diretriz Brasileira para o Cuidado e a Utilização de Animais em Atividades de Ensino ou de Pesquisa Científica (DBCA) issued by the CONCEA. If a renewal of the project is intended, the request must be submitted to the CEUA-ICB/USP secretary before the expiration of the current proposal. After this date, a new proposal must be prepared.

Espécie/Species	Linhagem/Strain	Sexo/Gender	Idade-Peso/ Age-Weight	Total
Felis Silvestris catus	*Felis Silvestris catus*	*Fêmea/female*	*5 anos/years*	*1*
Mus musculus	*C57BL/6*	*Macho/male*	*8 semanas/weeks*	*1200*
	Swiss	*Macho/male*	*8 semanas/weeks*	*80*
	Fos-2A-dsTVA	*Macho/male*	*8 Semanas/weeks*	*120*
	Swiss	*Fêmea/female*	*8 semanas/weeks*	*80*
	C57BL/6	*Fêmea/female*	*8 semanas/weeks*	*200*
	Balb/c	*Macho/male*	*8 semanas/weeks*	*400*
	Vglut2-ires-Cre Knock-in mice: Slc32a1<tm2(cre)Lowl>/J	*Macho/male*	*60 dias/days*	*60*
	Vgat-ires-Cre Knock-in mice: Slc17a6<tm2(cre)Lowl>/J	*Macho/male*	*60 dias/days*	*120*

São Paulo, 21 de março de 2017.

Prof. Dr. **Anderson de Sá Nunes**
Coordenador CEUA-ICB/USP

Eliane Aparecida Gomes de M. Nascimento
Secretário CEUA-ICB/USP

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RESUME

Titre : Etudes des circuits neuronaux organisant l'évitement actif instrumental et l'évitement contextuel non instrumental

Les mammifères, y compris les rongeurs, présentent un large éventail de comportements défensifs actifs, tels que l'évitement, ou passif, tel que l'immobilisation (le freezing) à des fins d'adaptation et de survie. La réaction d'évitement est une réaction apprise dans laquelle un individu prend le contrôle de situations dangereuses pour faire face aux menaces. Une forme d'évitement qui a été étudiée est l'évitement actif signalé, où les individus sont entraînés à éviter un environnement et fuient en réponse à un signal précédemment associé à un stimulus aversif. Il a été souligné que le Cortex préfrontal dorso-médian (CPFdm) joue un rôle important dans l'encodage de l'acquisition et de l'expression de freezing ainsi que dans les réponses d'évitement. Cependant, sa contribution à l'acquisition et à l'expression de comportements d'évitement n'est pas claire, et les circuits neuronaux du CPFdm qui gèrent ensemble les stratégies d'adaptation actives et passives, restent à découvrir. Pour répondre à cette question, nous avons développé un nouveau paradigme comportemental dans lequel une souris a la possibilité de se figer ou d'éviter un stimulus aversif en fonctions des contingences contextuelles. Premièrement, nous avons étudié le rôle de la voie entre le CPFdm et PAG dans l'évitement actif signalé, et sa relation avec le freezing. Nos résultats indiquent que (i) le CPFdm et le dl/lPAG sont activées lors du comportement d'évitement, (ii) et que l'inhibition optogénétique de cette voie bloquait l'acquisition de l'évitement conditionné. Une forme d'évitement non instrumental est également étudiée, dans laquelle l'individu apprend à éviter l'environnement aversif en utilisant uniquement des indices contextuels et affichant des comportements d'évaluation des risques à l'encontre de l'environnement dangereux. Il a été précédement démontré que dans cette situation, un circuit septohippocampale-hyptothalamique-tronc cérébrale est spécialement activé. Cette analyse a aussi révélé que le PMD devait être impliqué de manière critique dans l'évitement passif contextuel. Nous avons analysé l'influence de la modulation du PMD et de ses projections sur ses cibles principales, sur le processus d'expression et de reconsolidation de l'évitement passif contextuel. Nos résultats ont montré qu' une (i) voie septohippocampale-hyptothalamique-tronc cérébrale spécifique était impliquée dans notre paradigme d'evitement passif. (ii) De plus, l'inhibition du PMD lors d'une exposition au contexte aversif altère à la fois l'expression des comportements d'évitement et la reconsolidation de la mémoire. (iii)Enfin l'inhibition au niveau des terminaux du PMD altère l'expression et la reconsolidation de la mémoire dans le dlPAG et dans l'AMv. Les expériences de ce projet ont été faites grâce à l'analyse Fos, l'inhibition pharmacogénétique, et des outils optogénétiques.

Mots clés: Evitement actif. Evitement Passif. Cortex préfrontal. Hipotalamo. Optogénétique

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.

RÉSUMÉ DE LA THÈSE

Tout au long du règne animal, les individus présentent un répertoire de comportements défensifs liés à leurs besoins de survie, selon leur environnement. En effet, les animaux adoptent des stratégies défensives pour se protéger et / ou protéger leurs congénères contre les dangers environnementaux. Les mammifères, y compris les rongeurs, présentent un large éventail de comportements défensifs actifs, tels que les comportements d'évitement, ou passif, tel que le comportement d'immobilisation (le freezing). L'adaptation comprend la sélection de la stratégie défensive appropriée en tenant compte de ses coûts, de la menace et du contexte dans lequel elle se produit (Hofmann et Hay, 2018). C'est pourquoi lorsque le danger est évitable, des comportements défensifs actifs tels que l'évitement et la fuite sont adoptés (Ramirez et al., 2015; Blanchard et Blanchard, 1969). L'évitement est une réaction apprise dans laquelle un individu prend le contrôle de situations dangereuses pour faire face aux menaces. Une forme d'évitement qui est couramment étudié est l'évitement actif signalé, il s'agit d'une situation où les individus sont entraînés à éviter un environnement et s'échappent en réponse à un signal précédemment associé à un stimulus aversif (Moscarello and ledoux, 2013). Il existe aussi une forme d'évitement non instrumentale généralement étudiée, dans laquelle l'individu apprend à éviter l'environnement précédemment associé à un stimulus aversif en utilisant uniquement des indices contextuels et en affichant des comportements d'évaluation des risques à l'encontre de l'environnement dangereux.

L'évitement actif signalé est un phénomène impliquant des fonctions cognitives complexes. Il a été souligné que le Cortex préfrontal dorso-médian (CPFdm) joue un rôle important dans l'encodage de l'acquisition et de l'expression de freezing ainsi que dans les réponses d'évitement. Cependant, son implication dans l'acquisition et l'expression de comportements d'évitement n'est pas claire, et les circuits neuronaux du CPFdm qui gèrent ensemble les stratégies d'adaptation actives et passives, restent à découvrir. Pour répondre à cette question, nous avons développé un nouveau paradigme comportemental dans lequel une souris a la possibilité de se figer passivement à un stimulus aversif ou de l'éviter activement, ceci dépendant des contingences contextuelles. Dans la première partie de ce projet, nous avons étudié, à l'aide d'analyse immunohistochimique de Fos, d'enregistrements électrophysiologiques et de manipulations de circuits neuronaux utilisant des techniques

optogénétiques, le rôle de la voie entre le cortex préfrontal et la matière grise périaqueducale dans l'évitement actif signalé, et sa relation avec le freezing.

Dans un deuxième temps nous nous sommes intéressées à un paradigme d'évitement passif, qui donne la possibilité à l'animal d'entrer ou non dans un environnement familier associé à un évènement aversif (un choc). D'après une même étude de notre labo chez le rat (Viellard et al.), et une revue de la littérature de peur innée contextuelle, il était d'attrait de se pencher sur un circuit septohippocampal-hypothalamo-tronc cerebrale. Grace à ces données il a été révélé que le noyau prémammillaire dorsal (PMD) devait être impliqué de manière critique dans l'expression d'évitement contextuel et la reconsolidation de la mémoire aversive. En utilisant des outils immunohistochimique, d'inhibition pharmacogénétique, d'optogénétiques, nous avons analysé l'influence de la modulation du PMD et de ses principales cibles principales (à savoir, le PAG et le thalamus antéroventral), sur le processus d'expression et de reconsolidation de l'évitement passif contextuel.

Résultat partie I : Rôle de la voie préfrontale – tronc cérébrale médiant le comportement d'évitement actif instrumental.

Nos différentes études ont prouvé que notre nouveau paradigme permet aux animaux de répondre de manière passive (freezing), ou active (évitement) suivant le même stimulus conditionné. Ce paradigme, par sa complixité de l'exercice, exposé une hétérogénéité dans l'apprentissage de l'évitement, créant un groupe de bon esquiveur et de mauvais esquiveur. Par ailleurs cette hétérogénéité n'est pas révélée au niveau du freezing. Une étude de nage forcée (FST) a prouvé que ces différences n'était pas résultante du test en lui-même, mais était inhérente aux individus. Suivant une première étude d'immunohistochimie nous avons pu remarquer que le CPFdm et les sous-régions dorsolatérales et latérales de la matière grise périaqueducale (dl/lPAG) sont activées lors du comportement d'évitement. De plus, en marge avec les résultats dans la thèse de Dr. Suzana Khoder démontrant que, utilisant l'enregistrement unitaire électrophysiologique, une sous-population de neurones du CPFdm vers le dl/lPAG codant l'évitement mais pas le comportement de freezing, il a été verifié que, l'activation ou l'inhibition optogénétique de cette voie favorisait et bloquait l'acquisition de l'évitement conditionné. Enfin il a été décrit que l'apprentissage de l'évitement était associé au développement de la plasticité du CPFdm à des synapses dl/lPAG.

Résultat partie II : Rôle des voies hypothalamiques médiant l'évitement passive non instrumental.

A la suite de cette première partie nous avons étudié l'influence des circuits hypothalamique dans une situation d'évitement passive non instrumentalisé. Nos résultats, utilisant une technique de révélation de la protéine Fos, ont mis en évidence une voie septohippocampale-hypothalamique-tronc cérébrale spécifique impliquée dans la situation d'évitement passif. Cette voie n'est pas impliqué lorsque l'animal est confiné sans échappatoire dans un espace aversif. De plus, à l'aide d'outils pharmacogénétique, nous avons inhibé le PMD lors de l'exposition au contexte aversif après conditionnement, démontrant l'altération à la fois de l'expression des comportements d'évitement et la reconsolidation de la mémoire, durant la réexposition au même contexte le jour suivant. Enfin par le biais de l'illumination optogénétique au niveau des terminaux du PMD, nous avons conclu que l'inhibition dans le dlPAG et dans le AMv altère l'expression d'évitement et la reconsolidation de la mémoire.

Conclusion

A la fin de ce projet il a pu être démontré dans un premier temps, qu'un circuit du cortex préfrontal projetant vers la partie dorsolatéral du PAG, est spécifiquement impliqué dans la réponse d'évitement actif ; et dans un second temps, l'évitement passif est contrôlé en partie par un nucleus de l'hypothalamus postérieur , le PMD, ainsi que par ces deux sites de projections principales, l'AMv et le dlPAG. Nos résultats révèlent une dynamique neuronale très intéressante entre des réponses impliquant des fonctions cognitives plus complexes, comme l'apprentissage de l'évitement actif signalé dépendant d'une voie spécifique du cortex préfrontal vers le dlPAG. Des réponses reposant sur un apprentissage contextuel plus simple telles que l'évitement passif, en revanche, dépendent de sites hypothalamiques, qui par ailleurs, sont également impliqués de manière critique dans les comportements défensifs innés liés aux menaces prédatrices et sociales.

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List of abbreviations

AAV	Adeno-associated virus
ACC	Anterior cingulate cortex
AHN	Anterio hypothalamic nucleus
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMv	Ventral part of the anteromedial nucleus of the thalamus
ArchT	Archeorodhopsin
BA	Basal nucleus of amygdala
BLA	Basolateral nucleus of amygdala
CAV	Canine adenovirus
CCK	Cholecystokinin
CeA	Central nucleus of amygdala
CeL	Centrolateral nucleus of amygdala
CeM	Centromedial nucleus of amygdala
CS	Conditioned stimulus
CS-	Control stimulus (not paired with shock)
CS+	Conditioned stimulus (paired with shock)
dlPAG	Dorsolateral periaqueductal gray
dmPAG	Dorsomedial periaqueductal gray
dmPFC	Dorsomedial prefrontal cortex
DREADD	Designer receptors exclusively activated by designer drugs
GABA	Gamma-aminobutyric acid
GFP	Green Fluorescent Protein
hM4Di	Gi-coupled human M4 muscarinic DREADD receptor
IL	Infra limbic
IN	Interneuron
LA	Lateral nucleus of amygdala
LH	Lateral hypothalamus
LHAjd	Lateral hypothalamic area juxtadorsomedial region
lPAG	Lateral periaqueductal gray
MD	Medial segment of the Thalamus

MDTB	Mouse Defense Test Battery
mPFC	Medial prefrontal cortex
Nac	Nucleus accumbens
NacC	Nucleus accumbens core
NacS	Nucleus accumbens shell
NMDA	N-Methyl- d-aspartic acid
NYP	Neuropeptide Y
PAG	Periaqueductal gray
PCA	Principal Component Analysis
PFC	Prefrontal cortex
PL	Prelimbic cortex
PMD	Dorsal Premammillary nucleus
PN	Pyramidal neurons
PPN	Putative pyramidal neurons
PrCm	Medial precentral cortex
PTSD	Post-traumatic stress disorder
PV	Parvalbumin
RA	Risk assessment
SigA	Signalled active avoidance
SST	Somatostatin
SUBv	Ventral Subiculum
UR	Unconditioned response
US	Unconditioned stimulus
vHPC	Ventral hippocampus
VIP	Vaso active intestinal peptide
vlPAG	Ventrolateral periaqueductal gray
vlPFC	Ventrolateral prefrontal cortex
VMH	Ventral part of the medial hypothalamus
vmPFC	Ventromedial prefrontal cortex
VTA	Ventral tegmental area

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; Low 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).

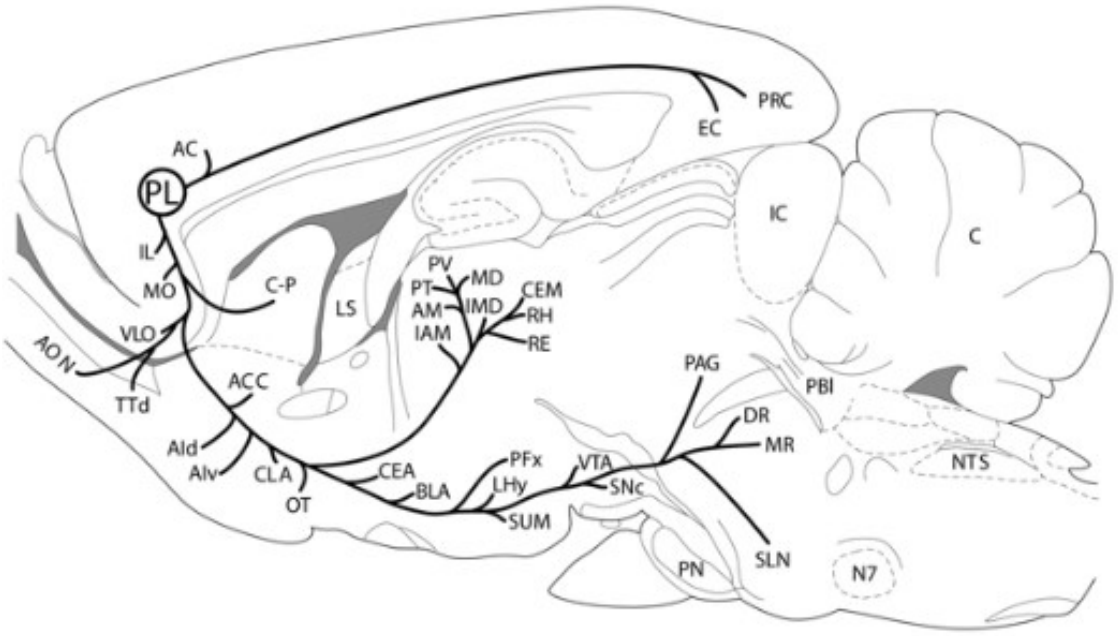

Figure 1 Schematic sagittal sections summarizing the main efferents projections of the PL in rats Sections are modified from the rat atlas of Paxinos and Franklin (Paxinos and Franklin, 2008)

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 nucleus (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).

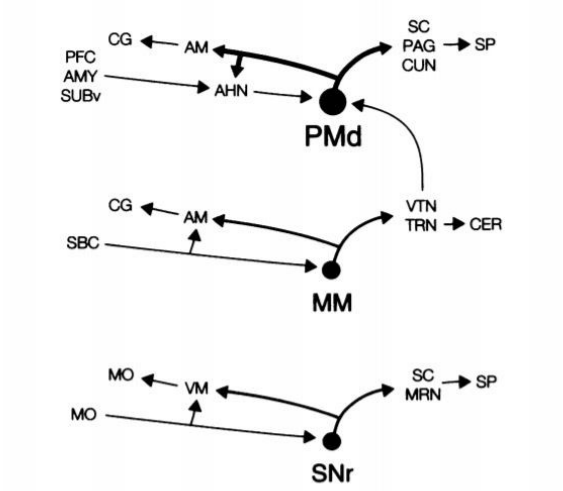

FIG. 4. Major input/output relations of the PMd, medial mammillary nucleus (MM), and reticular part of substantia nigra (SNr). Hypothetical pathway from the periaqueductal gray (PAG) to the spinal cord (SP) may be direct or indirect. AHN, anterior hypothalamic nucleus (n.); AM, anteromedial n. thalamus; 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)

Aims of the thesis

First part: Role of the prefrontal-brainstem pathway in mediating avoidance behavior

1. Establish the behavioral paradigm for SigAA, and determine the criteria for the good and bad avoiders;
2. Examine the fos activation pattern of the PAG, mPfC and amygdala
3. Inhibit the dmPFC-dlPAG pathway during avoidance using optogenetic to untangle whether the dmPFC-dlPAG pathway is involved in the expression and/or acquisition of avoidance behavior.

Second part: Hypothalamic pathways of shock-based Passive avoidance

1. Validate the passive avoidance paradigm and the Fos activation pattern of the septohippocampal-hypothalamic-brainstem pathway
2. Investigation of the role of the PMD in the expression and reconsolidation processes of contextual passive avoidance.
3. Inhibition of the projections from the PMD to its main target (the dorsal PAG and ventral part of the anteromedial thalamic nucleus) and study their influence on the expression and re-consolidation processes of contextual passive avoidance.

Note :

The results section is divided in two main parts, and each result part is preceded by its own material and methods section. The first part is devoted to the investigation of role of the prefrontal-brainstem pathway in mediating avoidance behavior, a work carried out in Dr. Herry's lab, at the university of Bordeaux. This part of the result section is my contribution to the work of Dr. Suzana Khoder's, that has been published in her thesis in november 2018. The manuscript of an article is under finalization before submission to a high profile journal where Suzana and I will be first co-author of the work. The second part of the results is a second project carried out in the university of São Paulo, in the lab of Prof. Newton Canteras during which we investigated Hypothalamic pathways of shock-based Passive avoidance.

I/ Role of the prefrontal-brainstem pathway in mediating avoidance behavior

Materials and methods

I. Animals

Animals SigAA paradigm: Male C57BL6/J mice (3 months old; Janvier) weighing 30-35 g at the time of surgery, were group-housed upon arrival in a 22°C colony room, on a 12-hours light/dark cycle (lights on at 7 a.m.) and were provided with food and water ad libitum. Mice were housed individually 3 days before surgeries and manipulated daily. Animals' experiments were carried out in accordance with standard ethical guidelines (European Communities Directive 86/60-EEC).

II. Experimental protocol

The apparatus is composed of two identical square compartments of 25cmx25cm. It is separated by a descending door linked to the Imetronic software. The sound and the shock are also monitored by the Imetronic software.

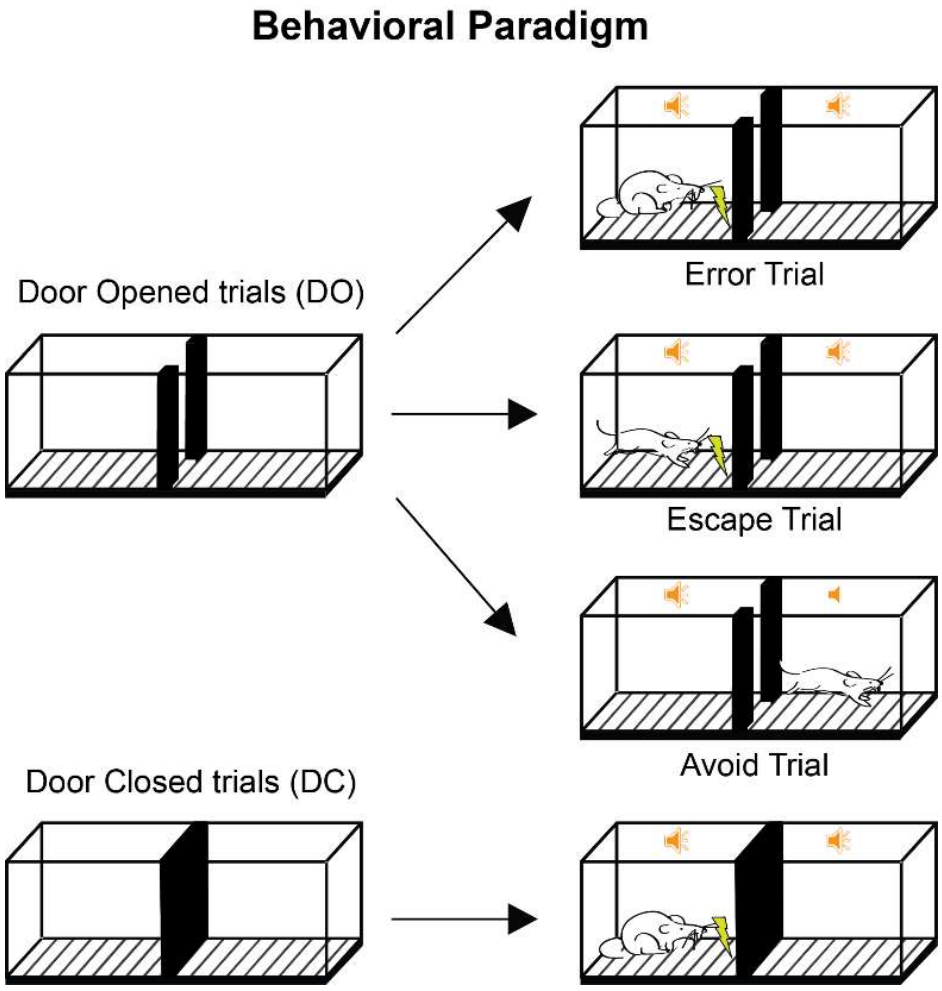

Figure 3 **Behavioral paradigm.** In the door opened trials (DO), 3 types of behavioral readouts were scored: **error trials** during which animals stayed during the whole CS^+ and the US delivery; **escape trials** during which mice crossed to the opposite compartment of a shuttle-box during the US and **avoid trials** during which animals crossed during the CS^+ and avoided the US. In door-closed trials (DC), **freezing** behavior was assessed during the sound presentation.

Avoidance learning protocol:

Habituation to context (*Figure 4*): On day 1, the mice were habituated to the shuttle box for 15 minutes.

Habituation to door and tones (*Figure 4*): On day 2, the mice were habituated to the door opening and different tones (7Hz pips for CS+, white noise for CS-). The different trials (CS-/CS+ door open and CS-/CS+ door closed) were presented in a shuffled manner 9 times each. The number of shuttles for each trial was counted.

Acquisition (*Figure 4*): on day 3 to 8, the mice performed a 6 day training session presenting the different trials : CS-/CS+ door open (followed by 4 sec footshock, for the CS+, 0,6 mA) and CS-/CS+ door closed (followed by 4 sec footshock for the CS+, 0,6 mA) were presented in a shuffled manner 15 times each. The animals were left 3 minutes freely exploring the environment at the beginning and at the end of each session. The following outcomes were counted (Figure 3): number of error trials, escape trials, avoid trials, and the freezing level.

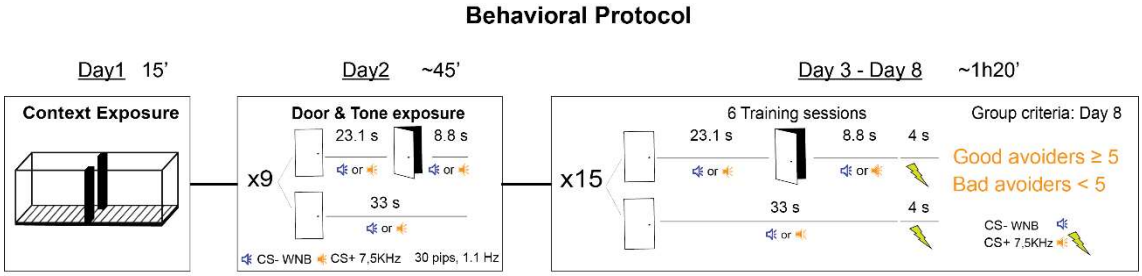

Figure 4 **Behavioral protocol** On day 1, mice were habituated during 15 min to the shuttle-box. On day 2, animals were habituated to the presentation of two sounds that were played in two contextual conditions : **(i)** door-closed trials (DC) during which the sound was played for 33 s , and **(ii)** door-opened trials (DO) during which 23.1 s following the sound's onset the door was slided-down (DO) and slided-up again 8.8 sec after. 9 trials of each of the DO and DC types of trials were played for both CS^- and CS^+ and the session lasted about 45 min. From day 3 to day 8, animals underwent 6 training sessions lasting each about 1h20min and during which the same type of trials than during day 2 were played except that the number of trials was increased to 15, and that CS^+ trials were followed by a 4 s shock in the DC condition. At day 8, animals were categorized into **Good** or **Bad avoiders** based on their behavioral avoidance scores.

III. Forced-swim test (FST)

Following avoidance training, Good and Bad avoiders underwent a FST session during which each mouse was individually placed in a cylindrical tank (50 cm height and 20 cm width) filled with clean tap water (24 ± 1 °C). Mice were forced to swim during 6 minutes. The first two minutes were considered as an acclimatization time and during the last 4 minutes, Behavioral Observation Research Interactive Software (BORIS) was used to process the recorded behavioral video allowing to score the total duration of immobility. Mice were scored to show immobility when they floated without struggling and making only those movements necessary to keep their heads above the water: namely moving only their hind paws but not front paws. In the end of the FST, mice were carefully dried before being returned to their home cages.

IV. Histological processing of Fos immunohistochemistry

Following the 6th session of training mice were divided into three groups and underwent a last 7th behavioral session. The first control group received, at a 7th behavioral session, only 15 CS- trials. The second and the third groups, which were respectively classified at session 6 as Bad and Good avoiders underwent a 7th behavioral session during which they received 15 trials of CS+ presentations without footshocks. A fourth group of naïve mice was also used as a control. Ninety minutes after the 6th behavioral session (not for the naïve mice), The animals were perfused after being deeply anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and perfused transcardially with a solution of 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and left overnight in a solution of 20% sucrose in 0.1 M phosphate buffer at 4 °C. The brains were then frozen, and 5 series of 40-µm-thick sections in the frontal plane were cut using a sliding microtome. One series of sections was processed for immunohistochemistry. The sections were incubated with anti-Fos antiserum raised in rabbit (Ab-5; Calbiochem) at a dilution of 1:20,000. The primary antiserum was detected using a variation of the avidin–biotin complex system. In brief, the sections were incubated for 90 min at room temperature in a solution of biotinylated goat anti-rabbit IgG (Vector Laboratories) and then placed in a mixed avidin–biotin horseradish

peroxidase (HRP) complex solution (ABC Elite Kit; Vector Laboratories) for 90 min. The black-blue peroxidase complex was visualized after a 5 min exposure to a chromogen solution containing 0.02% 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma) with 0.3% nickel ammonium sulfate in 0.05 M Tris buffer (pH 7.6), followed by a 20 min incubation in a chromogen solution containing hydrogen peroxide (1:3000). The reaction was stopped using potassium phosphate-buffered saline (KPBS; pH 7.4). The sections were mounted on gelatin-coated slides, dehydrated and cover slipped using DPX mounting media (Sigma). An adjacent series of sections was stained with thionin (Nissl stain) to serve as a reference series for cytoarchitectonic purposes. Images of the selected brain regions (PFC, PAG, amygdala) were generated using a Nikon Eclipse 80i (10 x magnification, Nikon Corporation, Chiyoda-Ku, Tokyo-To, Japan) microscope equipped with a Nikon digital camera (DXM1200F, Nikon Corporation). To quantify the density of the Fos-labeled cells, we first delineated the borders of the selected brain regions by referring to the reference (Nissl-stained) sections and the mouse brain atlas (Paxinos, 2008). Then, the Fos-labeled cells were counted. Only darkly labeled oval nuclei that fell within the borders of a region of interest were counted. The density of Fos labeling was determined by dividing the number of Fos-immunoreactive cells by the area of the region of interest. Both the cell counting and area measurements were performed with the aid of a computer program (Image-Pro Plus, version 4.5.1; Media Cybernetics, Silver Spring, MD, USA). Cell densities were obtained on both sides of the brain and were averaged for each mouse.

V. Viral injections and optogenetics

For specific optogenetic manipulation of the dmPFC-dl/lPAG pathway during behavior we used C57BL6/J wild-type mice. Animals were bilaterally injected with glass pipettes (tip diameter 10-20 µm) connected to a picospritzer (Parker Hannifin Corporation; ~ 0.2 µL per hemisphere) with a cocktail of Cav2-Cre, HSV-Cre retrograde virus and AAV-hSyn-mCherry in the dl/lPAG at the following coordinates relative to bregma: −4.50 mm AP; ± 0.5 ML; −1.45 DV from dura. The same animals received also an injection of AAV9-FLEXArchT-GFP, or AAV5-FLEX-GFP (UNC Vector Core Facility) in the dmPFC at the

following coordinates relative to bregma: +1.8 mm AP; ± 0.40 ML; −1.3 DV from dura. Following 4 weeks of recovery from injections, mice were implanted with optic fibers in the two hemisphere at the following coordinates relative to bregma: +1.8 mm AP; ± 0.55 mm ML; −1.15 mm DV from dura; lowered at an angle of 10°.

Optical stimulation

A laser generating a continuous yellow light at 593 nm or a blue light at 473nm (DPSSL lasers) was connected to a 200 µm diameter optic fibre patch cable (Plexon) and calibrated to produce a fibre tip irradiance of approximately 16-18 mW. Computer software (Imetronic) controlled the timing and duration of laser pulses. Laser stimulations were delivered to the dmPFC to transiently activate or inactivate pyramidal projection of the dmPFC neurons to the dlPAG.

VI. Statistical analysis

Analyses were performed with Matlab, Graphpad Prism and Statview. For all datasets normality was tested using the Shapiro-Wilk normality test ($\alpha < 0.05$) to determine whether parametric or non-parametric analyses were required. Parametric analyses included t. tests and one- and two-way repeated-measures ANOVA followed by Bonferroni's, or Fisher's PLSD (for behavior only) multiple comparison post-hoc test if a significant main effect or interaction was observed. For parametric data, correlation analyses were made using Pearson's correlation. If datasets did not meet normality assumptions non-parametric analyses were used (mainly non-parametric Mann-Whitney test). If significance was observed, these non-parametric analyses were followed by Dunn's multiple comparison post hoc tests. For non-parametric data, correlation analyses were made using Spearman's correlation. Apart from t. tests, the asterisks in the figures represent the P-values of post hoc tests corresponding to the following values $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ based on mean ± S.E.M.

Results

I. Behavior

In order to investigate the neural circuits underlying freezing and avoidance responses, a novel paradigm was developed. It would create the possibility to condition the animal with the same CS to both an open door situation (DO) where the animal has the possibility to escape/avoid a shock, and a closed door situation (DC) where the animal would only have the choice to freeze waiting for the shock.

The results showed that this behavioral paradigm generated a difference of learning profile within the group of trained mice (Figure 5**. a, b**). The animals where then categorized in two groups, the bad avoiders and the good avoiders. The bad avoiders (55%) showed a deficit in learning performances as they couldn't reach 5/15 avoidances after the 6th day of training. The good avoider group (33%) (**Figure 6d)** was selected following two parameters. First, they had to reach 5/15 avoidances at the end of the 6th session, second, they had to discriminate between the CS+ and the CS-, according to the avoidance discrimination index (**Figure 6a**). Notably, 13% of the animals (**Figure 6d.**), successfully avoided during the CS+ but also during the CS-. These animals were called the generalizers and were excluded from the analysis as they had a very low discrimination index (**Figure 6b,d**). Another indicator of learning was the latency to avoid. The good learners learned to avoid the CS+ faster than the CS- (Figure 5**d**). Interestingly, the bad avoiders did not learn to avoid, however, the escape latency decreases across the sessions (Figure 5**c.**). Importantly, the escape latency of the good avoiders also decreased (Figure 5**, c.**), but was not different from the bad avoiders. It indicates that the good and the bad avoiders had the same escape kinetics.

Regarding freezing expression, the Bad avoiders froze significantly more than the Good avoiders during CS+ presentation in DC condition. However they had a significant difference of freezing level between the CS+ and the CS- (Figure 5**e**). It indicates that they would discriminate between the two CSs. During the DO condition they showed no difference of freezing before and after the door opening (Figure 5**f)**.

For the Good avoiders, they also discriminated the CS+ and CS- in terms of freezing level during the DC condition (Figure 5**g**). And as they would learn to change their strategy during DO condition and avoid, as expected, they would have a significant reduced freezing level after the opening of the door (Figure 5**h.**).

It should be noted that freezing discrimination was rapidly acquired in both groups, as the difference is clear from the first day of training. However the avoidance acquisition was a longer process, as the good avoiders would learn to avoid and discriminate between the CSs at late training stage (Figure 5**b., Figure 6a.**).

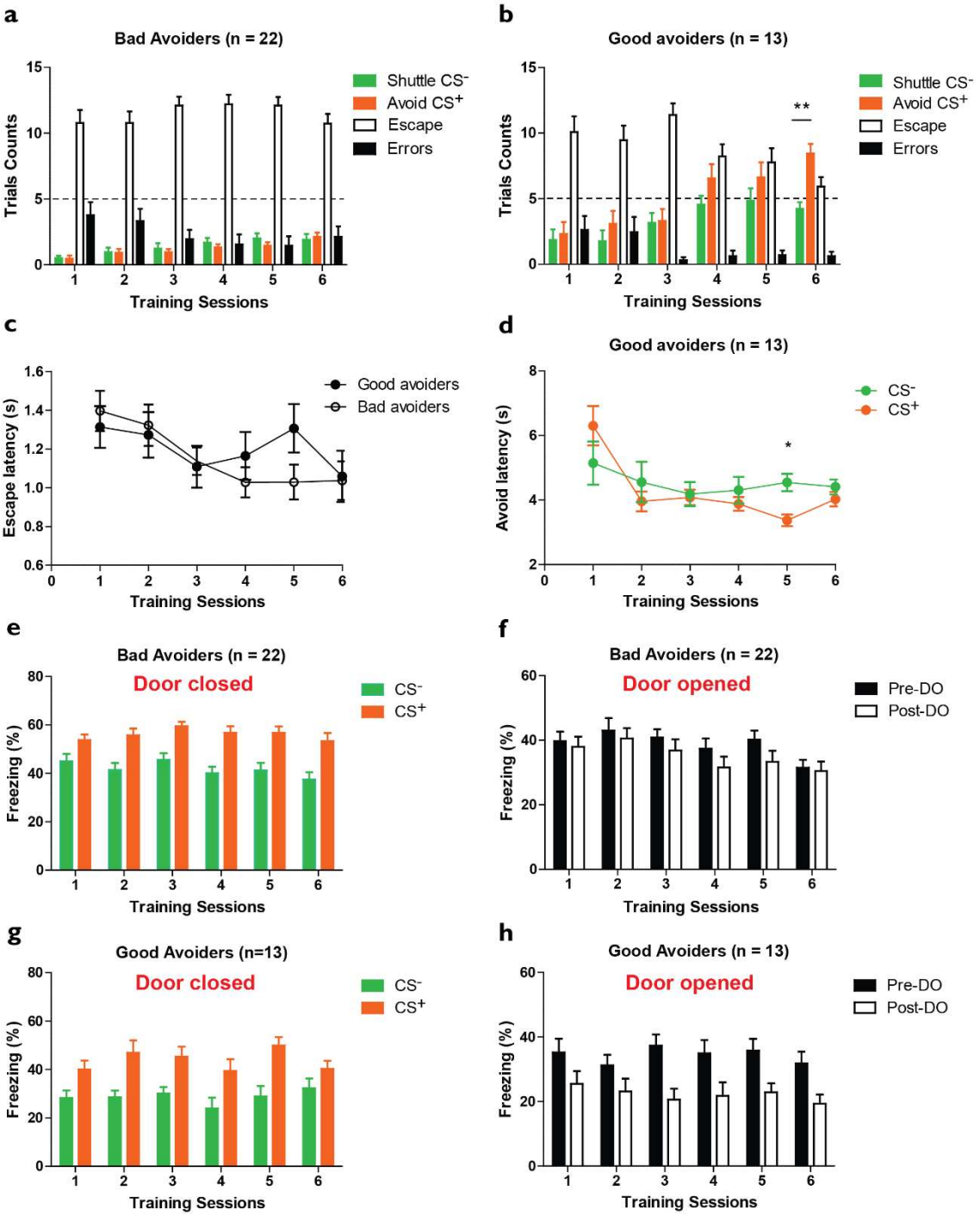

Figure 5 **Behavioral characterization of Good and Bad avoiders**. **a.** Trial counts (shuttle CS^-, avoid CS^+, escape, errors) across 6 training sessions in **Bad avoiders** (n = 22) (two-way repeated measures ANOVA; group: $F_{(3,420)} = 104.5$, $p < 0.0001$, training session: $F_{(5,420)} = 0.50$, $p = 0.77$, group x training session: $F_{(15,420)} = 3.98$, $p < 0.0001$). **b.** Trial counts (shuttle CS^-, avoid CS^+, escape, errors) across 6 training sessions in Good avoiders (n =13) (two-way repeated measures ANOVA; group: $F_{(3,240)} = 28.43$, $p < 0.0001$, training session: $F_{(5,240)} = 1.13$, $p = 0.34$, group x training session: $F_{(15,240)} = 8.83$, $p < 0.0001$, and $p < 0.01$). **c.** Escape latency (s) for both **Good** and **Bad avoiders** across 6 training sessions (two-way repeated measures ANOVA; group: $F_{(1,184)} = 0.54$, $p = 0.45$, training session: $F_{(5,184)} = 2.55$, $p = 0.02$, group x training session: $F_{(5,184)} = 0.82$, $p = 0.53$). **d.** Avoid latencies during DO trials for both CS^+ and CS^- trials in Good avoiders (n = 13) (two-way

repeated measures ANOVA; group: $F_{(1,114)} = 1.56$, $p = 0.21$, training session: $F_{(5,114)} = 7.03$, $p < 0.0001$, group x training session: F (5,114) = 2.45, $p = 0.03$, * $p < 0.05$). **e.** Averaged freezing behavior in **Bad avoiders** (n = 22) across training sessions for both CS^+ and CS^- at door-closed trials (two-way repeated measures ANOVA; group: $F_{(1,210)} = 30.80$, $p < 0.0001$, training session: $F_{(5,210)} = 1.96$, $p = 0.08$, group x training session: $F_{(5,210)} = 0.76$, $p = 0.57$). **f.** Averaged freezing behavior in **Bad avoiders** across training before and after door opening (8.8s pre-DO and post-DO) (two-way repeated measures ANOVA; group: $F_{(1,210)} = 2.05$, $p = 0.15$, training session: $F_{(5,210)} = 3.68$, $p = 0.003$, group x training session: $F_{(5,210)} = 0.34$, $p = 0.88$). **g.** Averaged freezing behavior in **Good avoiders** (n = 13) across training sessions for both CS^+ and CS^- at door-closed trials (two-way repeated measures ANOVA; group: $F_{(1,120)} = 17.82$, $p = 0.0003$, training session: $F_{(5,120)} = 1.53$, $p = 0.18$, group x training session: $F_{(5,120)} = 1.04$, $p = 0.39$). **h.** Averaged freezing behavior in **Good avoiders** across training before and after door opening (8.8 s pre-DO and post-DO) (two-way repeated measures ANOVA; group: $F_{(1,120)} = 23.67$, $p < 0.0001$, training session: $F_{(5,120)} = 0.47$, $p = 0.79$, group x training session: $F_{(5,120)} = 0.37$, $p = 0.86$).

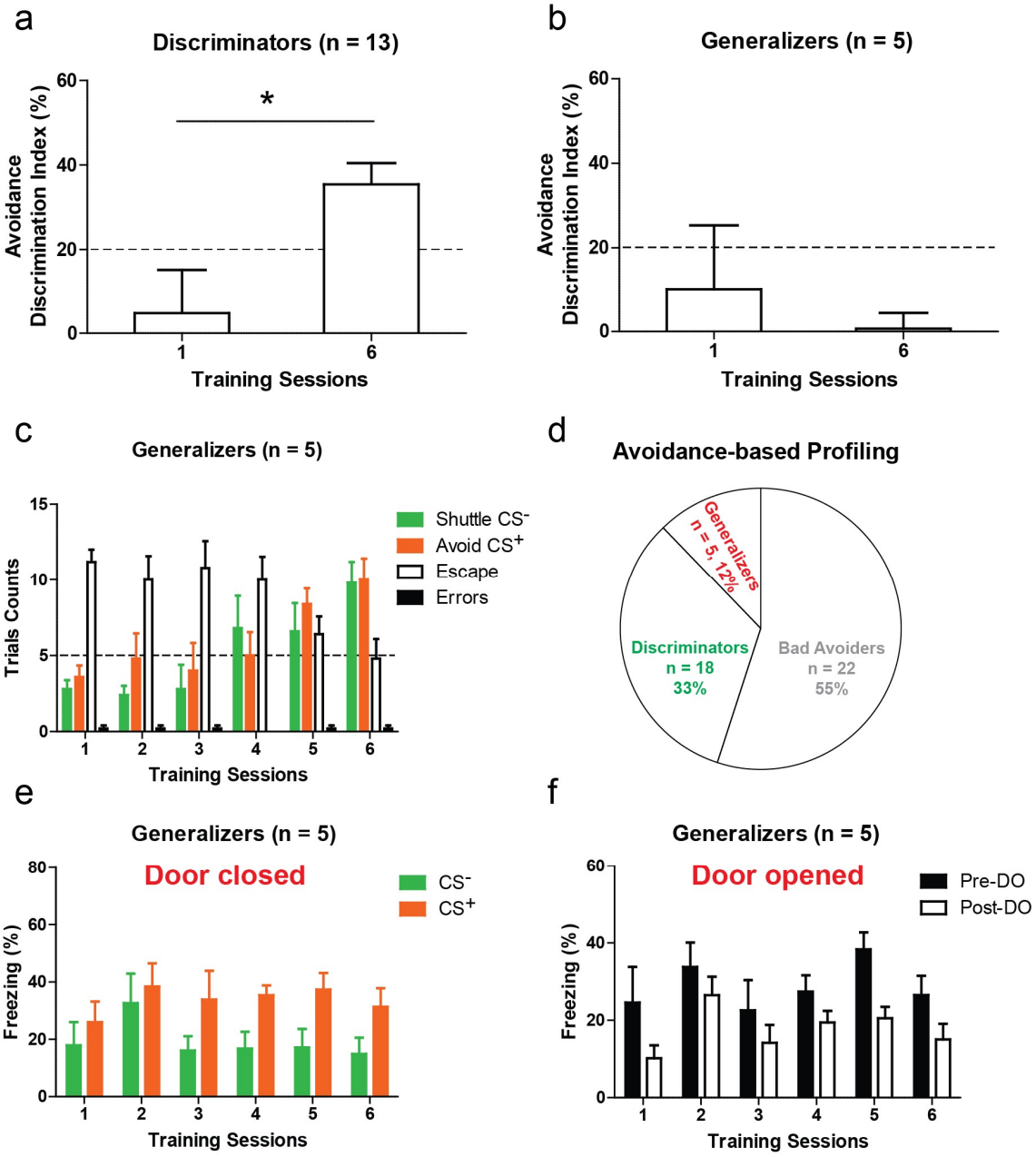

Figure 6 A subset of Good avoiders generalized between CS⁻ and CS⁺ during avoidance

a. Avoidance discrimination index calculated as following ((Avoidance counts CS⁺) - (avoidance counts CS⁻)) / ((Avoidance counts CS⁺) + (Avoidance counts CS⁻ +1)) for **Good avoiders** discriminators (paired t. test: t = 2.81, p = 0.015) and generalizers in panel **b.** (paired t.test: t = 0.57, p = 0.59) at first and sixth training sessions. The dashed line at 20% represents the cut-off that we consider to classify mice as generalizers versus discriminators. **c.** Trial counts (shuttle CS⁻, avoid CS⁺, escape, errors) across 6 training sessions in generalizers (n = 5) (two-way repeated measures ANOVA; group: $F_{(3, 80)}$ = 17.90, p < 0.0001, training session: $F_{(5, 80)}$ = 2.30, p = 0.052, group x training session: $F_{(15, 80)}$ = 6.96, p < 0.0001). **d.** Pie-chart representative of avoidance-based profiles for the 40 mice tested. **Bad avoiders** represent 55 % (n = 22), **Good avoiders** discriminators 33 % (n = 13) and generalizers 12 % (n = 5). **e.** Averaged freezing behavior in generalizers (n = 5) across training sessions for both CS⁺ and CS⁻ at DC trials. (Two-way repeated measures

ANOVA; group: $F_{(1, 40)} = 5.36$, $p = 0.04$, training session: $F_{(5, 40)} = 1.24$, $p = 0.30$, group x training session: $F_{(5, 40)} = 0.47$, $p = 0.78$). **f.** Averaged freezing behavior in generalizers across training before and after door opening (8.8 s pre-DO and post-DO) (two-way repeated measures ANOVA; group: $F_{(1, 40)} = 6.05$, $p = 0.03$, training session: $F_{(5, 40)} = 2.80$, $p = 0.02$, group x training session: $F_{(5, 40)} = 0.40$, $p = 0.83$).

In summary, we developed a novel behavioral paradigm allowing a mouse to acquire and perform discriminative passive (freezing) and active (avoidance) behavior to a single conditioned stimulus depending on contextual contingencies. The kinetics of acquisition of both behaviors were dissimilar; discriminative freezing being acquired very rapidly as compared to a progressive acquisition of discriminative avoidance. In terms of our behavioral paradigm, two categories of mice were identified:

- Bad avoiders: acquired discriminative freezing very rapidly but did not acquire discriminative avoidance.

- Good avoiders: acquired discriminative freezing early during training and discriminative avoidance progressively with training.

II. Forced Swim Test

Our behavioral paradigm resulted in Good and Bad avoidance learners. Although this may simply represent a phenotypic trait, we were concerned that a repetitive footshock experience in DC trials might promote learned helplessness behaviors, which could be manifested by a lack of avoidance responses, like in the Bad avoiders group. Therefore we conducted a standard test that would model learned helplessness: the forced swim test (FST) (Figure 7**a.**).

The results of this test illustrate the fact that good and bad avoiders show no difference of swimming/immobility strategy during the force swim test (Figure 7**c.**). They show the same amount of immobility and there is not correlation with their performance during the 6th session of the avoidance training (**Figure 7d**). Interestingly, If we take into account two animals that were excluded from the analysis (a generalizer, and an animal that received every shock without escaping), there will be a difference between the two groups (Figure 7**b.**). In fact the former spent the entire time swimming without stopping and the latter stayed immobile in the water and was rescued from drowning. These two animals are representing a very small percentage of our study, and the FST was a good indicator to exclude them.

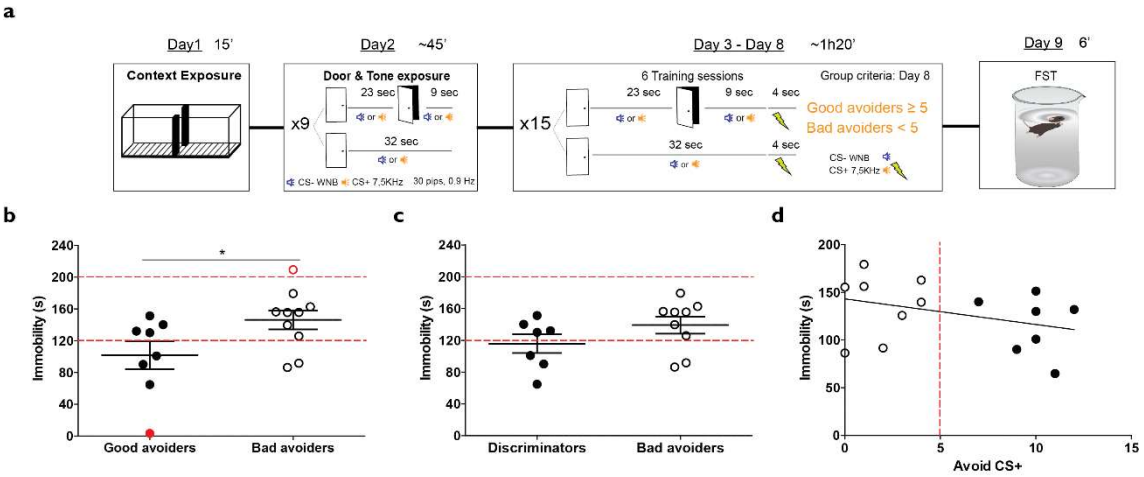

Figure 7 **Bad and Good avoiders do not differ in the forced swim test**

a. Mice undergoing the avoidance behavioral protocol for 8 days (see Figure 4) were classified into **Good** and **Bad avoiders** and were exposed one day later to the forced swim test (FST) during 6 minutes. **b.** Time spent immobile during the 4 last minutes of the FST for **Good** and **Bad avoiders**. Filled red circle represent a generalizer and empty red circle represent a mouse with a learned helplessness profile which spent all the 4 minutes immobile that was about to drown at the end of the session (unpaired t. test: $t = 2.19$, $p = 0.04$). **c.** Time spent immobile during the 4 last minutes of the FST for **Good** and **Bad avoiders** excluding the learned helplessness profile mouse (unpaired t. test: $t = 1.47$, $p = 0.16$). **d.** Correlation between the number of avoidance to the CS^+ and the time spent immobile during the 4 last minutes of the FST (Spearman correlation $r = -0.34$, $p = 0.18$). Filled circles concern the **Good avoiders** and empty circles concern the **Bad avoiders**. The horizontal dashed lines represent the lower and upper limits of immobility time range of a control group of mice exposed to the same FST protocol (Kara, et al., 2014; Kara, et al., 2016). The vertical dashed red line represents the threshold separating **Bad** and **Good avoiders**.

The disparity of performance of the animals is not reflected in the forced swim test. The fact that repeated shocks during training did not make them change strategies of coping during the forced swim test, can exclude the fact that Bad avoiders present learned helplessness.

So far the disparity in the behavioral profiles observed (Good versus Bad learners) is an interesting phenomenon as it allows investigating the underlying neuronal mechanisms and to perform loss and gain of function optogenetic experiments.

III. C-fos Immunoreactivity

After determining the behavioral dynamic of our paradigm, we studied the fos pattern of different regions of interest right after the end of the training sessions. Our interest in this analysis was to see the difference of Fos staining pattern between the bad and the good avoiders. We modified the protocol by separating the animals in four groups, and testing them only to one specific trial contingency (see Forced-swim test).

The results showed an upregulation of the fos protein in the dmPFC in the good avoiders group (Table 1, Figure 8a., Figure 9top panel). This group was different from the Bad avoiders and the controls. When looking at the activation pattern of the vmPFC, the results showed no difference between the groups (Table 1, Figure 8b.). The Good avoiders also showed a difference with the control groups in the dlPAGc (Table 1., Figure 8d., Figure 9 bottom Panel). However the group was not different from the Bad avoiders. Other structures like the the dm,l, vlPAG and the BLA showed no differences in fos staining between the groups (Table 1, Figure 8c, e, f , g, h).

Structures	Atlas level	Groups				F(3,18) ; p
		Home Cage (n=6)	CS- (n=6)	Bad Avoiders (n=5)	Good Avoiders (n=5)	
dmPFC	1,94	539,95 ± 49,41	576,7 ± 56,04	353,03± 53,41	913,28 ± 127,11 °,*,**	8,98 ; 0,0007
vmPFC		294,35 ± 14,69	372,59 ± 6,51	116,56 ± 9,46	439,34 ± 28,27	2,37 ; 0,1043
BLA ant	-1,2	269,85 ± 38,57	278,48 ± 58,14	345,23 ± 64,74	391,21 ± 97,05	2,41 ; 0,1005
BLA post	-1,82	122,71 ± 43,5	142,07 ± 41,9	153,73 ± 42,62	208,06 ± 36,04	2,83 ; 0,0675
BA post		144,54 ± 47,55	157,64 ± 43,76	194,68 ± 38,15	225,99 ± 85,77	2,69 ; 0,0771
LA post		94,64 ± 25,43	124,77 ± 22,1	85,93 ± 47,29	181,56 ± 60,51	2,48 ; 0,0938
PAGdmr	-4,48	171,29 ± 33,11	153,23 ± 39,19	244,17 ± 38,55	285,49 ± 35,47	2,47 ; 0,0948
PAGdlr		102,43 ± 21,38	76,95 ± 13,94	93,71 ± 22,33	110,78 ± 12,56	0,65 ; 0,5934
PAGlr		314,34 ± 57,58	231,19 ± 27,28	286,33 ± 60,07	290,73 ± 42,93	0,58 ; 0,6384
PAGvlr		245,8 ± 67,75	196,19 ± 78,52	196,75 ± 50,51	234,01 ± 173,35	0,39 ; 0,7649
PAGdmc	-4,84	56,81 ± 7,1	64,43 ± 15,88	129,59 ± 33,08	138,6 ± 40,64	2,8 ; 0,0698
PAGdlc		48,34 ± 17,74	25,45 ± 15,34	49,16 ± 21,59	101,88 ± 31,14 °	3,92 ; 0,0258
PAGlc		253,34 ± 54,01	244,58 ± 41,23	220,87 ± 48,18	338,34 ± 46,63	1,05 ; 0,396
PAGvlc		149,12 ± 37,16	127,39 ± 21,5	123,61 ± 22,64	203,71 ± 35,81	1,38 ; 0,2808

Bonferroni/Dunn Post-Hoc test: Comparisons of all groups to Good Avoiders group are not significant unless the corresponding p-value is less than 0,0083.

° Different from Home Cage (HC)
* Different from CS-
** Different from Bad Avoiders

Table 1 C-fos immunostaining in Bad and Good Avoiders. C-fos immunoreactivity cell counts/mm² in the prefrontal cortex, amygdala and PAG of Bad and Good avoiders following 6 avoidance training sessions. dmPFC includes ACC and PL, vmPFC includes IL. **Ant:** anterior, **post:** posterior, **r:** rostral and **c:** caudal.

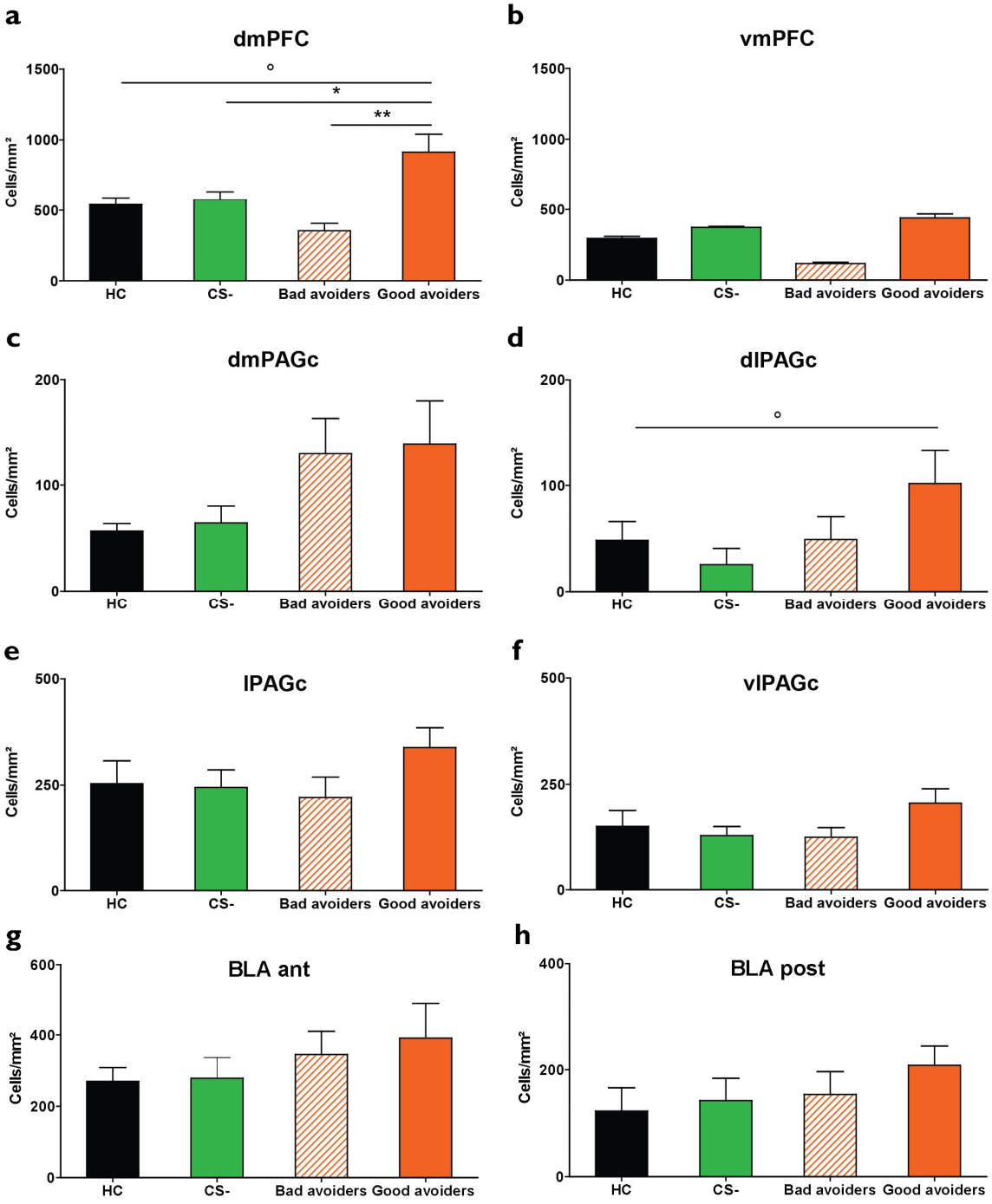

Figure 8 C-fos is expressed in the dmPFC and dlPAG of Good avoiders. Quantification of c-fos expression in home cage controls (HC), mice exposed to CS^-, **Bad** and **Good avoiders** exposed to CS^+ in the dmPFC (**a**), vmPFC (**b**), caudal dmPAG (dmPAGc) (**c**), caudal dlPAG (dlPAGc) (**d**), caudal lPAG (lPAGc) (**e**), caudal vlPAG (vlPAGc) (**f**), BLA ant (**g**) and BLA post (**h**).

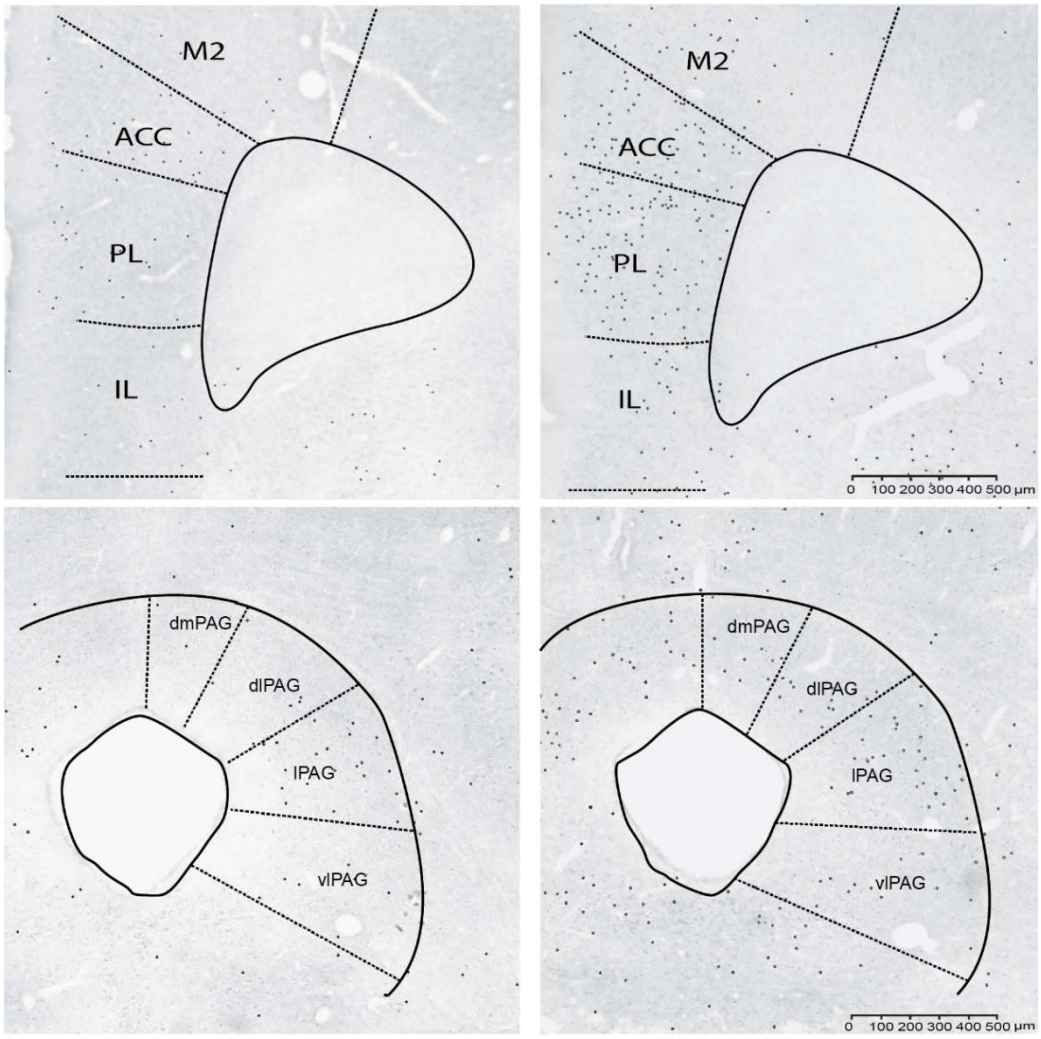

Figure 9 **C-fos immunostaining in Bad and Good avoiders.** Representative examples of c-fos staining in the **prefrontal cortex** (top panels) and the **PAG** (bottom panels) of a **Good avoider** (left column) compared to a **Bad avoider** (right column)

The present Fos study, established on our behavioral paradigm provided important information about subregional activations at the level of two structures. the dmPFC and the PAG. As expected, it suggests that it is rather the dmPFC, (and not the vmPFC) and the dlPAG which are activated during avoidance learning. Our data describe that the lack of learning in Bad avoiders is indeed correlated to a lower activation of the dmPFC. These data strengthen the optogenetic results on the activation of the dmPFC to dlPAG pathway promoting avoidance in Bad avoiders.

IV. Optogenetic manipulation

After validating the differences of fos activation between the Good and the Bad avoiders in the dlPAG and the dmPFC, we wanted to inhibit the dmPFC to dlPAG projections in order to understand its influence in the acquisition and expression of avoidance learning.

As we had previously optically activated the dmPFC neurons projecting to the dlPAG showing an improvement in the Bad avoiders performances after the training sessions, we wanted to see whether the inhibition of the pathway would impair the avoidance performances of the good avoiders, and make them bad avoiders.

According to our results, the optogenetic stimulation of the good avoiders the day after the 6th session had no effect on the avoidance level of the animals, and there was no difference with the GFP control group (Figure 10**b.**). This result let us think that inhibiting the dmPFC-dlPAG pathway did not affect animals that had already learnt the task. Regarding the latency to avoid, the good avoiders did not show differences in their latency to avoid after the stimulation (Figure 10**d.**). The discrimination between the CS+ and the CS- was also not impaired (Figure 10**e.**).

Interestingly, the inhibition of the pathway did not induce differences with the control groups in terms of freezing expression during the DC trials (Figure 10**a, b.**). Furthermore, in neither the shock nor the avoidance trials the animals reduced or increased their freezing level (Figure 10**c, d**.).

To conclude, the inhibition of the dmPFC-dlPAG pathway does not alter the performance of the good learners after they have learned the task. These results could indicate that the dmpFC to dlPAG pathway is not involved in the expression of active avoidance.

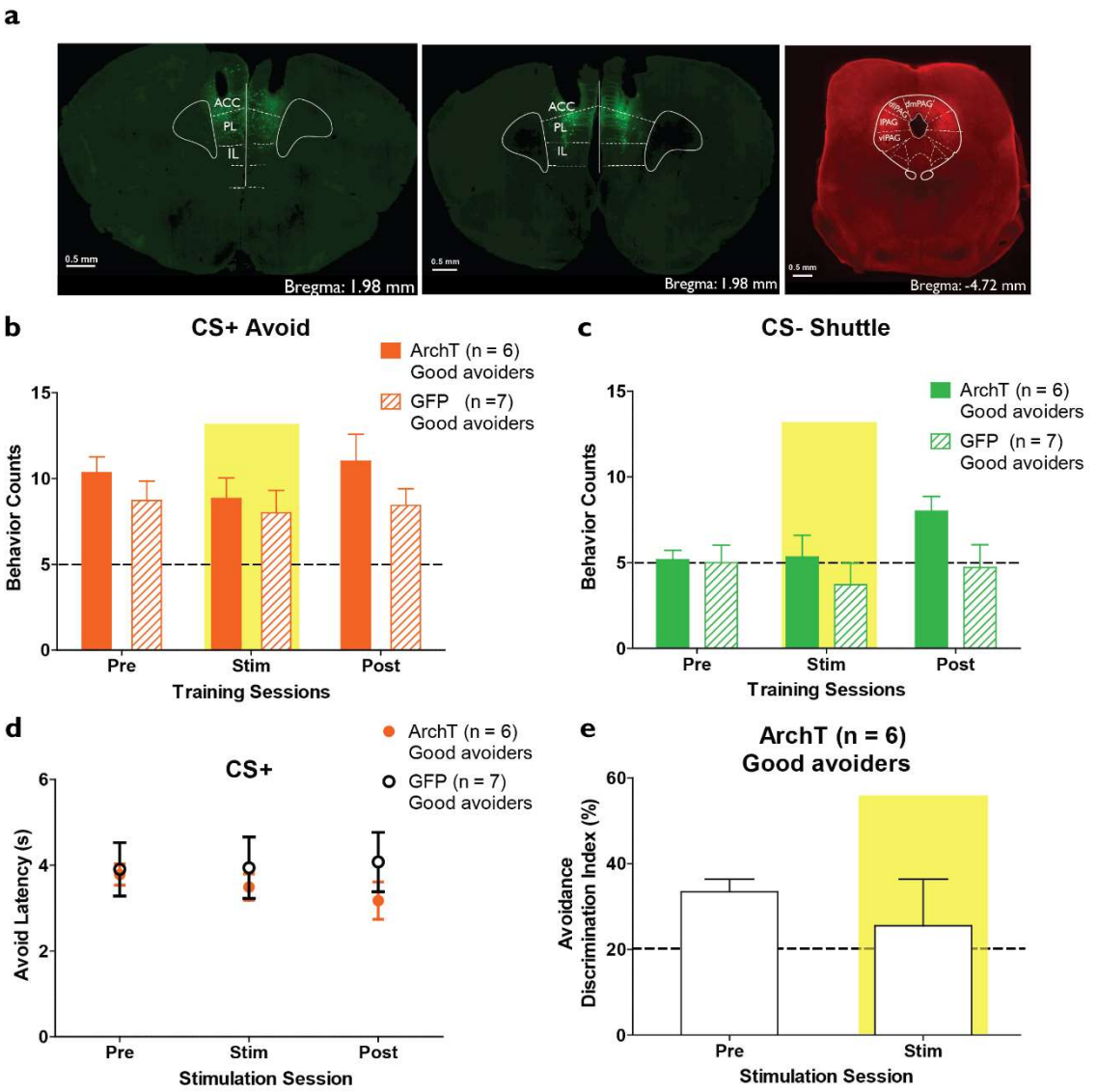

Figure 10 **Optogenetic inhibition of the dmPFC-dl/lPAG pathway does not impair avoidance expression**
a. Left panels: Photomicrographs of the fiber implantation and infection of the dmPFC to dlPAG pyramidal neurons in the PL with a (left) GFP and (right) ArchT cre dependent virus; **right panel** : photomicrograph of the retrograde cav-cre virus in the dl/lPAG **b.** CS^+ avoid counts at pre-stimulation, second stimulation and second post-stimulation sessions in two groups of **Good avoiders** infected with ArchT or GFP. (Two-way repeated measures ANOVA; group: $F_{(1, 22)} = 2.50$, $p = 0.14$, training session: $F_{(2, 22)} = 0.74$, $p = 0.48$, group x training session: $F_{(2, 22)} = 0.28$, $p = 0.75$). **c.** CS^- shuttle counts at pre-stimulation, second stimulation and second post-stimulation sessions in two groups of **Good avoiders**: ArchT and GFP groups. (Two-way repeated measures ANOVA; group: $F_{(1, 22)} = 3.01$, $p = 0.11$, training session: $F_{(2, 22)} = 1.56$, $p = 0.23$, group x training session: $F_{(2, 22)} = 1.08$, $p = 0.35$). **c.** Mean avoidance latency to CS^+ trials in two groups of **Good avoiders** expressing ArchT or GFP during the second stimulation session, the pre-stimulation and the post-stimulation sessions (two-way repeated measures ANOVA; group: $F_{(1,33)} = 1.13$, $p = 0.29$, training session: $F_{(2,33)} = 0.07$, $p = 0.92$, group x training session: $F_{(2,33)} = 0.24$, $p = 0.78$). **d.** Avoidance discrimination index in ArchT **Good avoiders** mice during the pre-stimulation session and the second stimulation session (paired t-test: $t = 0.63$, $p = 0.55$).

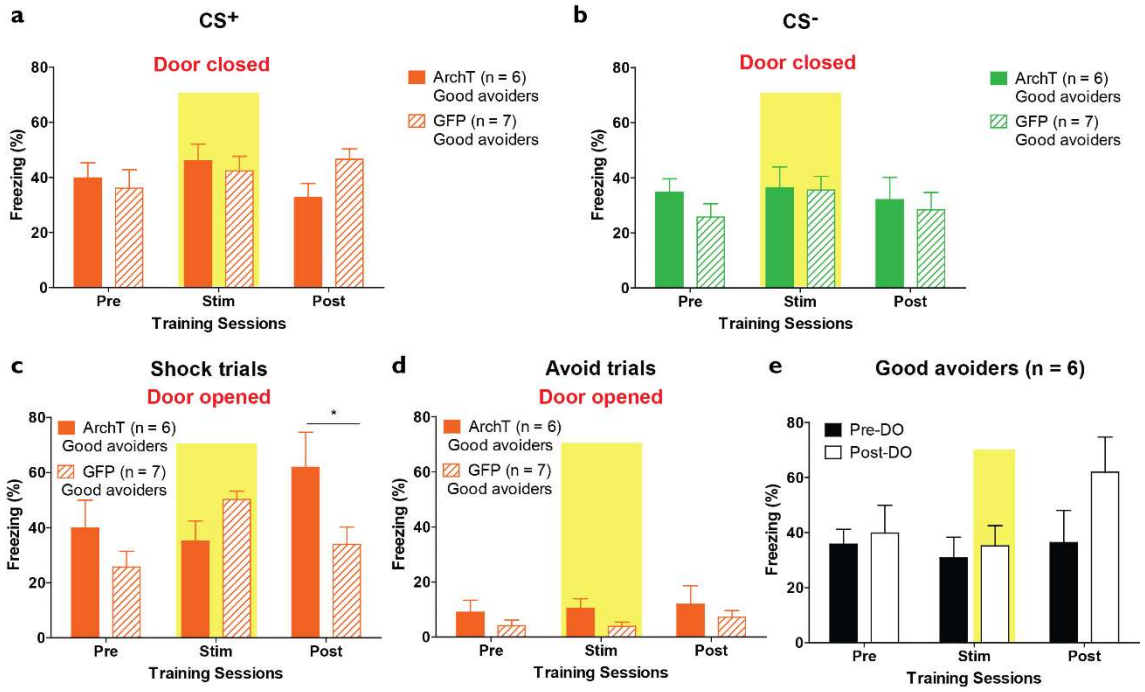

Figure 11 **Optogenetic inhibition of the dmPFC-dl/lPAG pathway does not impair freezing expression**
CS^+ -evoked freezing (across 15 trials) **(a),** and CS^- -evoked freezing (across 15 trials) **(b)** at pre-stimulation, second stimulation and second post-stimulation sessions in two groups of good avoiders: ArchT and GFP groups (panel **a**: two-way repeated measures ANOVA; group: $F_{(1, 22)} = 0.10$, $p = 0.75$, training session: $F_{(2, 22)} = 1.66$, $p = 0.21$, group x training session: $F_{(2, 22)} = 4.12$, $p = 0.03$, and panel **b**: two-way repeated measures ANOVA; group: $F_{(1, 26)} = 0.47$, $p = 0.50$, training session: $F_{(2, 26)} = 0.91$, $p = 0.41$, group x training session: $F_{(2, 26)} = 0.35$, $p = 0.70$). CS^+ -evoked freezing during (i) the interval between the door opening and the shock delivery (shock trials) **(c)** and (ii) the interval between the door opening and avoidance response (avoid trials) **(d)** at pre-stimulation, second stimulation and second post-stimulation sessions in ArchT and GFP control mice (panel **c,** two-way repeated measures ANOVA; group: $F_{(1, 32)} = 2.15$, $p = 0.15$, training session: $F_{(2, 32)} = 2.07$, $p = 0.14$, group x training session: $F_{(2, 32)} = 4.24$, $p = 0.02$, and panel **d**: two-way repeated measures ANOVA; group: $F_{(1, 33)} = 3.37$, $p = 0.07$, training session: $F_{(2, 33)} = 0.39$, $p = 0.67$, group x training session: $F_{(2, 33)} = 0.04$, $p = 0.95$). e. CS^+ -evoked freezing during the interval between the DO and the shock delivery (post-DO) and the same interval of time (namely 8.8 seconds) preceding the door opening (pre-DO) at pre-stimulation, second stimulation and second post-stimulation sessions in **Good avoiders** (two-way repeated measures ANOVA; group: $F_{(1, 28)} = 0.001$, $p = 0.96$, training session: $F_{(2, 28)} = 0.05$, $p = 0.94$, group x training session: $F_{(2, 28)} = 0.34$, $p = 0.70$).

Our next step was to focus our interest on the influence of the inhibition of the dmPFC-dlPAG pathway at early learning stage. As inhibiting the pathway had no effect on avoidance after training, it ruled out the role of this pathway in the expression of active avoidance, however, an inhibition during training will give us more information about its involvement in learning. We then inhibited the pathway in DO condition during the six days of training. To do so we simplified the paradigm by exposing the animals to the opened door condition only (DO+, DO-), as this classical active avoidance paradigm is known to generate a bigger proportion of good avoiders (Figure 12a.).

When inhibiting the pathway at early learning stage, the stimulated group was not able to avoid during the CS+ at the end of the 6th day, whereas the control group reached significantly higher avoidance rates (Figure 12c, d.). Interestingly, when left with no stimulation during six more days of training, the ArchT group stayed with bad performances, incapable to learn the task, while the control group kept improving (Figure 12c, d.). Regarding the freezing expression level, there is no significative difference between the stimulated sessions and the non stimulated sessions (Figure 12e, f.). However the ArchT group displayed significantly more freezing than the control group. This result cannot be explained by the stimulation but more likely by the fact that the ArchT group avoided less, ergo froze more, as shown with the bad learners (Figure 5).

In conclusion, the inhibition of the dmPFC to dlPAG pathway at early training stage abolished avoidance learning but did not affect conditioned freezing behavior.

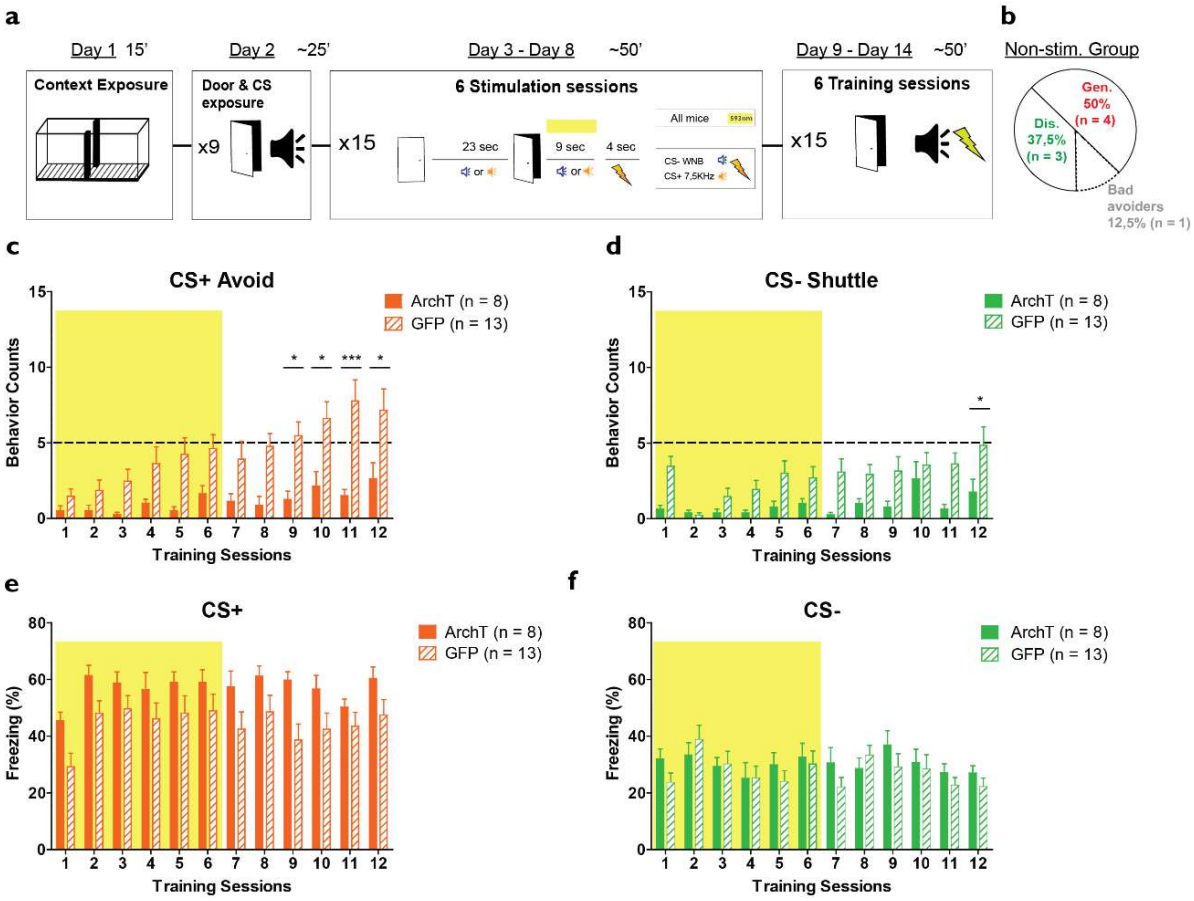

Figure 12 **Inhibition of the dmPFC-dl/lPAG pathway abolished avoidance learning**

a. Adapted behavioral protocol. On Day 1, mice were habituated during 15 min to the shuttle-box. On Day 2, animals were habituated to the presentation of the CS^- and CS^+ during opened condition (DO) only. After 23.1 seconds following the sound's onset, the door was slided-down (DO) and slided-up again 8.8 seconds after. 9 trials of CS^- and CS^+ were played. From Day 3 to Day 8, animals underwent 6 training during which the same type of trials were played except that the number of trials was increased to 15 CS^+ followed by a 4 s shock if the animal did not escape or avoid, and the yellow laser was turned on continuously for 9 seconds following door opening. From day 9 till 14 animals underwent the same training sessions except that no laser stimulation was delivered. **b.** Avoidance-based profiling after 6 sessions of training with no laser stimulation (non-stimulated group). **c.** CS^+ avoidance counts (two-way repeated measures ANOVA; group: $F_{(1, 209)} = 10.06$, $p = 0.005$, training session: $F_{(11, 209)} = 7.62$, $p < 0.0001$, group x training session: $F_{(11, 209)} = 2.42$, $p = 0.007$). **d.** CS^- shuttles counts (two-way repeated measures ANOVA; group: $F_{(1, 209)} = 7.72$, $p = 0.01$, training session: $F_{(11, 209)} = 3.91$, $p < 0.0001$, group x training session: $F_{(11, 209)} = 1.38$, $p = 0.18$,). **e, f.** Averaged freezing behavior during pre-door opening CS^+ trials (**e**: two-way repeated measures ANOVA; group: $F_{(1, 209)} = 4.19$, $p = 0.05$, training session: $F_{(11, 209)} = 4.27$, $p < 0.0001$, group x training session: $F_{(11, 209)} = 0.61$, $p = 0.81$), and CS^- trials (**f**: two-way repeated measures ANOVA; group: $F_{(1, 209)} = 0.35$, $p = 0.55$, training session: $F_{(11, 209)} = 2.72$, $p = 0.002$, group x training session: $F_{(11, 209)} = 1.22$, $p = 0.27$).

In summary, the optogenetic inhibition of dmPFC-dl/lPAG doesn't impair avoidance behavior in already good avoiders, but abolished the capacity to learn the task when the animals are repeatedly inhibited at early stage.

Altogether with the fos data, and the data on the optogenetic activation of the pathway in bad avoiders promoting active avoidance, we can propose that the dmPFC to dlPAG pathway promotes active avoidance but doesn't seem to be involved in its expression, nor in freezing. It is also supported with the electrophysiological data, showing a specific activation of the dmPFC projecting cells during avoidance.

II/ Hypothalamic pathways of shock-based Passive avoidance

Materials and methods

I. Animals

FOS expression experiments: Male C57BL6/J mice (3 months old) weighing 30-35 g were used in these experiments. There were housed the same way as previously described for the SigAA experiments.

Pharmacogenetic, optogenetic and electrophysiological experiments: CCK-Cre transgenic male mice (3-month-old, Jackson Laboratories) were used in these experiments. These animals express the Cre protein under control of the cholecystokinin promoter (CCK). Interestingly, this animal is ideal for manipulating the PMD, since this nucleus differs from neighboring structures by the abundant expression of CCK. There were housed the same way as above. we aimed at inactivating the PMD using pharmacogenetic tools. The PMD is a very small hypothalamic site, and the use of conventional forms of pharmacological inhibition would spread to other neighboring areas, rendering very difficult to ascertain the specific roles of the PMD in passive contextual avoidance. To circumscribe, as much as possible, the inactivation to the PMD, we took advantage to the fact that PMD cells present a characteristic expression of CCK (cholecystokinin)peptides, differing from the neighboring structures.

II. Experimental protocols

A/ Compared conditions Paradigm

The experimental apparatus consists of a safe cage (25x25x25 cm) with a door, connected to a 30cm corridor with access to a second cage (conditioning cage), with the same dimensions as the safe cage. The whole experimental apparatus is made of acrylic. The conditioning cage has a floor with a grid composed of steel bars spaced 7 mm and connected to a current generator (Insight).

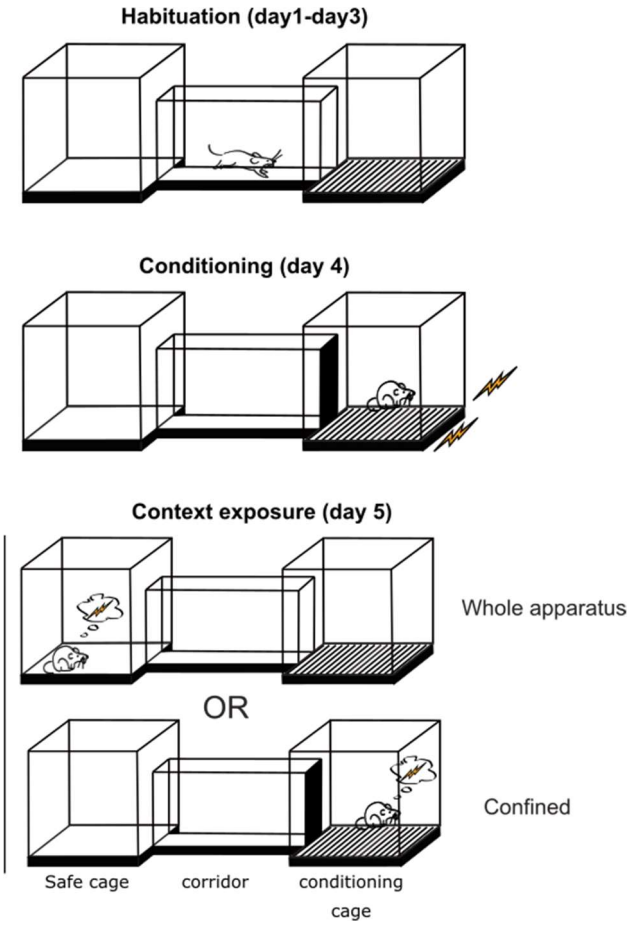

Figure 13 Behavioral protocol for the study of Fos expression in the contextual non-instrumental avoidance.

For three days, each animal was habituated to this experimental apparatus, where the animals were placed in the safe cage, allowing the animal to explore the entire experimental apparatus for 10 minutes. On the 4th day, the animals were placed in the apparatus, and when they entered the conditioning cage the door was shut, after two minutes of habituation they received five footshocks (0.6mA, for 1 second, following a random triggering pattern) and stayed in this box for an additional 2 minutes. The control groups had exactly the same treatment without receiving footshocks (not conditioned). The next day, four groups of animals were tested, namely:

Group 1 (Free+) : Animals that received shocks on day 4 were placed in the safe cage (Figure 13 panel "whole apparatus") with an access to the whole apparatus, including the conditioning cage and were observed for 8 minutes.

Group 2 (Confined+): Animals that received shocks on day 4 were confined in the conditioning cage for 8 minutes (Figure 13 panel "confined").

Group 3 (Free-) : unconditioned naive animals that were placed in the safe cage with access to the conditioning cage, and were observed for 8 minutes (Figure 13 panel "whole apparatus").

Group 4 (Confined-): unconditioned naive animals that were confined in the conditioning cage for 8 minutes (Figure 13 panel "confined").

B/ Passive avoidance Paradigm: PMD study

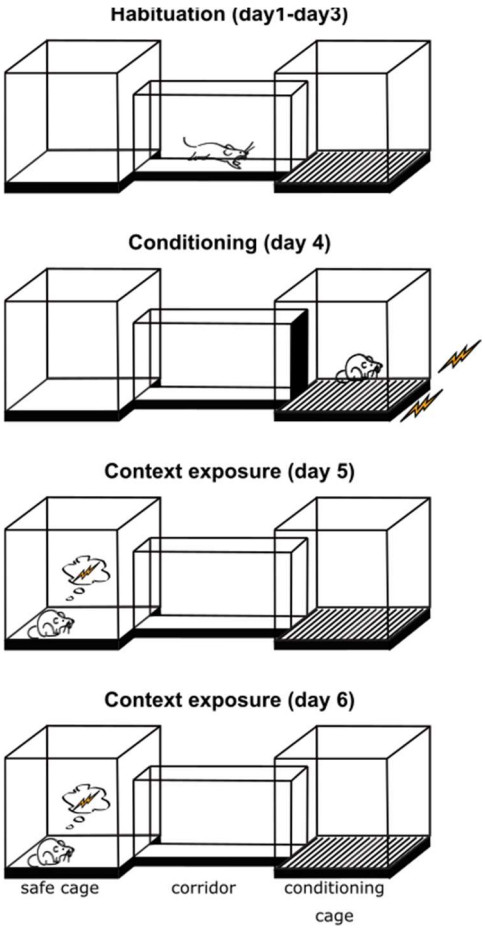

Figure 14 Behavioral protocol for the study of the role of the PMD in the contextual non-instrumental avoidance.

For the experiments of the PMD modulations, the animals were conditioned the same way as in the other paradigm (see *"compared conditions paradigm*"). On day 5, the

animals were placed in the safe cage with access to the conditioning cage and were observed for 8 minutes. On Day 6, the different groups were exposed again to the same context for 8 minutes.

Pharmacogenetic experiments: on day 5 the animals injected with an inhibitory DREADD virus (HM4D (Gi)) were administered a dose of 300µl of CNO (Clozapine *N*-oxide) solution, 30min before the context exposure. To measure the putative interoceptive stimulus effect of clozapine, the control group was also administered CNO but were injected with a non DREADD GFP virus (. On day 6, none of the groups received treatment. (Figure 19)

Optogenetic experiments: on day 5, both groups (Halorodopsin and GFP) were continuously stimulated with yellow light (wavelength of 589nm) for 8 minutes, when entering the conditioning apparatus. On day 6, the animals were not stimulated with light (Figure 24, Figure 26).

Generalisation experiment: The animals encountered the same procedure as in the *"passive avoidance paradigm"* but were exposed prior to the conditioning apparatus, to a neutral open field (60cmx40cmx20cm) for 5 minutes. They were exposed during the three days of habituation, and the two days after the conditioning day (Figure 22).

III. Behavioral analysis

Animals were filmed and data analyzed later by a trained observer using the ethological analysis software The Observer (Noldus). The counted behaviors where the ones below (see Figure 15) The behavioral analysis was done during the last day of habituation, the conditioning phase and the next day in the test of contextual responses. The analysis involved spatiotemporal (for contextual responses) and behavioral (for conditioning and contextual responses) measurements. Spatiotemporal measurements are related to the time (in seconds) the animal spent in the safe cage, corridor, or conditioning box. Behavioral data were processed in terms of duration (8 min per session).

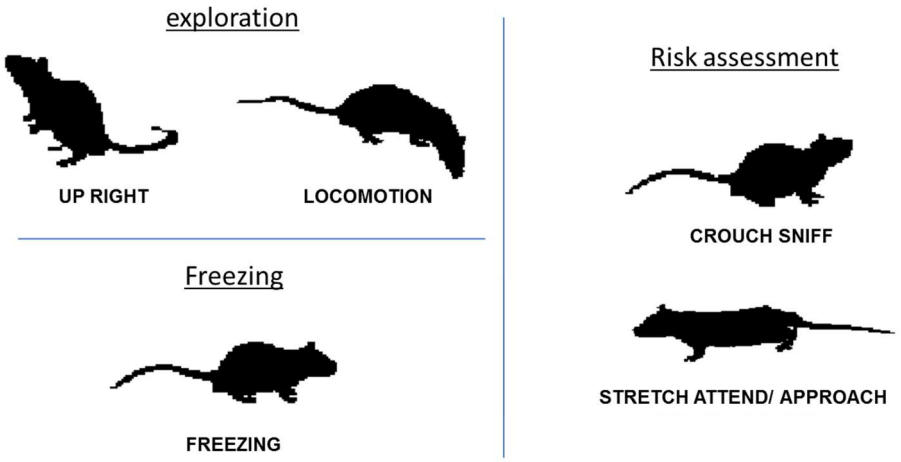

Figure 15 Behavioral features analysed during experiments The following behavioral responses were categorized: "Freezing" - the animal remained completely immobile in a freezing state except for the breathing movements; "Crouch sniff" - the animal remained still, with arched back, making movements with the head smelling and scanning the environment; "Stretch Attend Posture" - the animal extended the head and part of the body forward, kept the tail elevated, but did move; "Stretch Approach" - the animal retained the same anterior posture, but moved forward; "Up right position" - the animal was standing with its hind and front legs extended and supported on the walls of the apparatus; "Locomotion" -the animal moved more than 1cm with arched back;

IV. Histological processing of Fos immunohistochemistry

90 minutes following the exposure to the aversive context, the animals were anesthetized and perfused following the same technique as the first part. *(see Histological processing of Fos immunohistochemistry Part I).*

V. surgeries and optogenetics

Viral injection surgery: Following the same procedures described in the SigAA paradigm, we made bilateral injection in the PMD (coordinates: -2.43 mm antero posterior, -/+ 0.3 medio lateral, -5.4 ventral to the dura), where cre-dependent virus to express DREADD was injected in CCK-IRES-CRE mice.

Optical fiber implantation surgery: Three weeks after viral infection, using the same surgical and stereotaxic procedures previously described, cranial holes were drilled at the following stereotaxic coordinates + -2.3 mm anterior-posterior from the bregma and +/- 0.3 mm medial-lateral. For the inhibition of PMD projections, bilateral optic fibers were

implanted either in the dlPAG (-4.2 mm anterior-posterior from the bregma and + 0.5 (8°angle) mm medial-lateral, -1.35 mm ventral to the dura-mater); or in the Anterior thalamus (-0.7 mm anterior-posterior from the bregma and +/- 1.4 (10°angle) mm medial-lateral, -3.35 mm ventral to the dura-mater). After surgery, mice were allowed to recover for at least 7 days. They were handled daily during the three days preceding the behavioral tests to habituate them to the connection procedure.

Pharmacogenetics inhibition: To circumscribe the DREADD expression in the PMD we used CCK-Cre transgenic male mice (Jackson Laboratories), which express the Cre protein under control of the cholecystokinin promoter (CCK), and applied CRE-dependent virus for the expression of HM4D (Gi) in the PMD to be responsive to the inactivation with CNO.

Optical stimulation (see material and method part I): Terminals were inhibited by a continuous yellow light at 593 nm using a laser (DPSSL lasers) that was connected to a 200 µm diameter optic fibre patch cable (Thorlab) and calibrated to produce a fibre tip irradiance of approximately 16-18 mW. The animals were illuminated by yellow light while entering the apparatus to immediately block any possible contextual cue gathering.

VI. Statistical analysis

(see Statistical analysis in part I)

Results

I. Behavior analysis of the non-instrumental contextual avoidance paradigm.

In this part of the project we developed a paradigm where the animals, after being conditioned to a shock would be placed, depending on the group, in different conditions for fear retrieval. As shown in Figure 16b. the **Free-** group explored the entire apparatus and spent 217±23.1 s in the footshock chamber. In contrast, the **Free+** group did not enter the footshock chamber and stayed 587± 22.9 s in the safe cage and 28±4.9 s in the corridor (Figure 16b.). Looking at the behaviors, the **Free+** group (Figure 16a.) showed a small amount of freezing but showed a lot of risk assessment (i.e., "crouch-sniff" and "stretch postures") (Figure 16a.). Moreover, during the test period, the **Confined-** group explored fearlessly the conditioning chamber (Figure 16a.). The **Confined+** group, on the contrary, expressed freezing behavior and risk assessment during the exposure to the shock chamber (Figure 16a, c.).

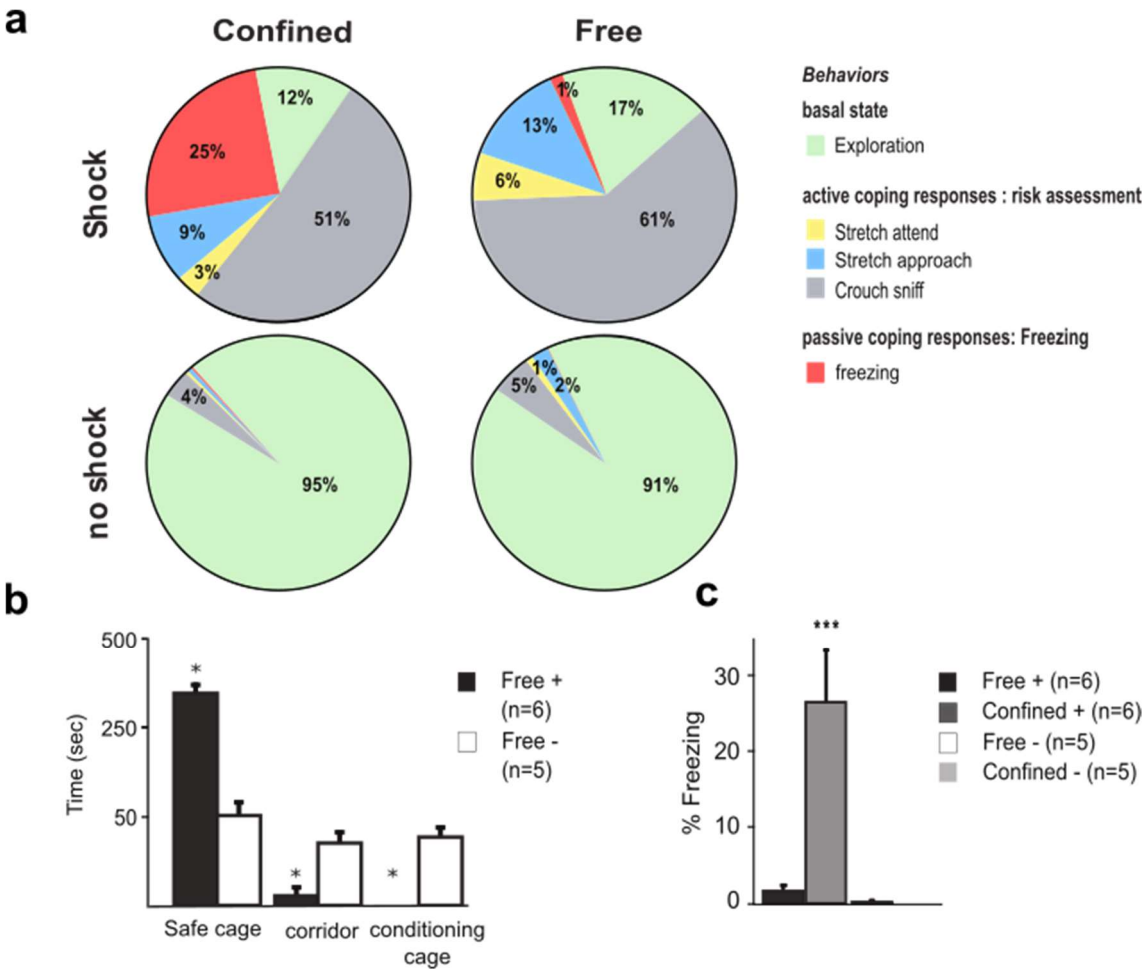

Figure 16. Behavior expression strategies differ depending on the fear recall exposure conditions. **a.** Pie charts of the behaviors counted during context exposure of: **Confined+** (top left panel), **Free+** (top right panel), **Confined-** (bottom left panel) and **Free-** (bottom right panel) groups. **b.** Comparison of the spatiotemporal measurements between **Free+** and **Free-** there is a difference in group*spatiotemporal distribution interaction: two-way ANOVA $F_{(1,8)}$=48.423 p>0.0001. **c.** Comparison of freezing expression level among the groups: ANOVA $F_{(3,16)}$=13.909 p<0.0001; Fisher PLSD post hoc: ***p<0.001 different from all groups.

These results indicate that specific behaviors are expressed during fear retrieval according to different exposure situations. Even though the animals have been conditioned the same way, the one in a free condition will express more risk assessment behaviors, and will not enter the conditioning cage, whereas the animals in a closed situation will express more freezing.

II. Analysis of Fos expression in the non-instrumental contextual avoidance paradigm.

After understanding the behavioral pattern of the defense responses toward different exposure conditions, we analysed the Fos upregulation of the structures of our interest. The selected areas for evaluation of the Fos protein expression were based on a previous study (Viellard et al. 2016), where during passive contextual avoidance we showed, in rats, the mobilization of the circuit formed by the ventral Subiculum (SUBv), lateral septum (LS), juxta-dorsomedial part of the lateral hypothalamus (LHAjd) and the dorsal premammillary (PMD). In addition, we investigated the expression of Fos in the dorsomedial, dorsolateral, lateral and ventrolateral sectors of the PAG. Our results show an upregulation of fos expression for the **Free+** group in the SUBv, the LS, the LHAjd, the PMD, the dmPAG, and the lPAG different form all of the other groups (**Figure 17a, b, Figure 18**). The Fos expression of the vlPAG is different between the **Free+** and the **Confined+** group but the **Free+** group is not different from its control (**Free-**). The **Free+** group also differs in Fos expression in the dlPAG from both control groups (**Free-, Confined-**), but not from the **Confined+** group. (**Figure 17b.**)

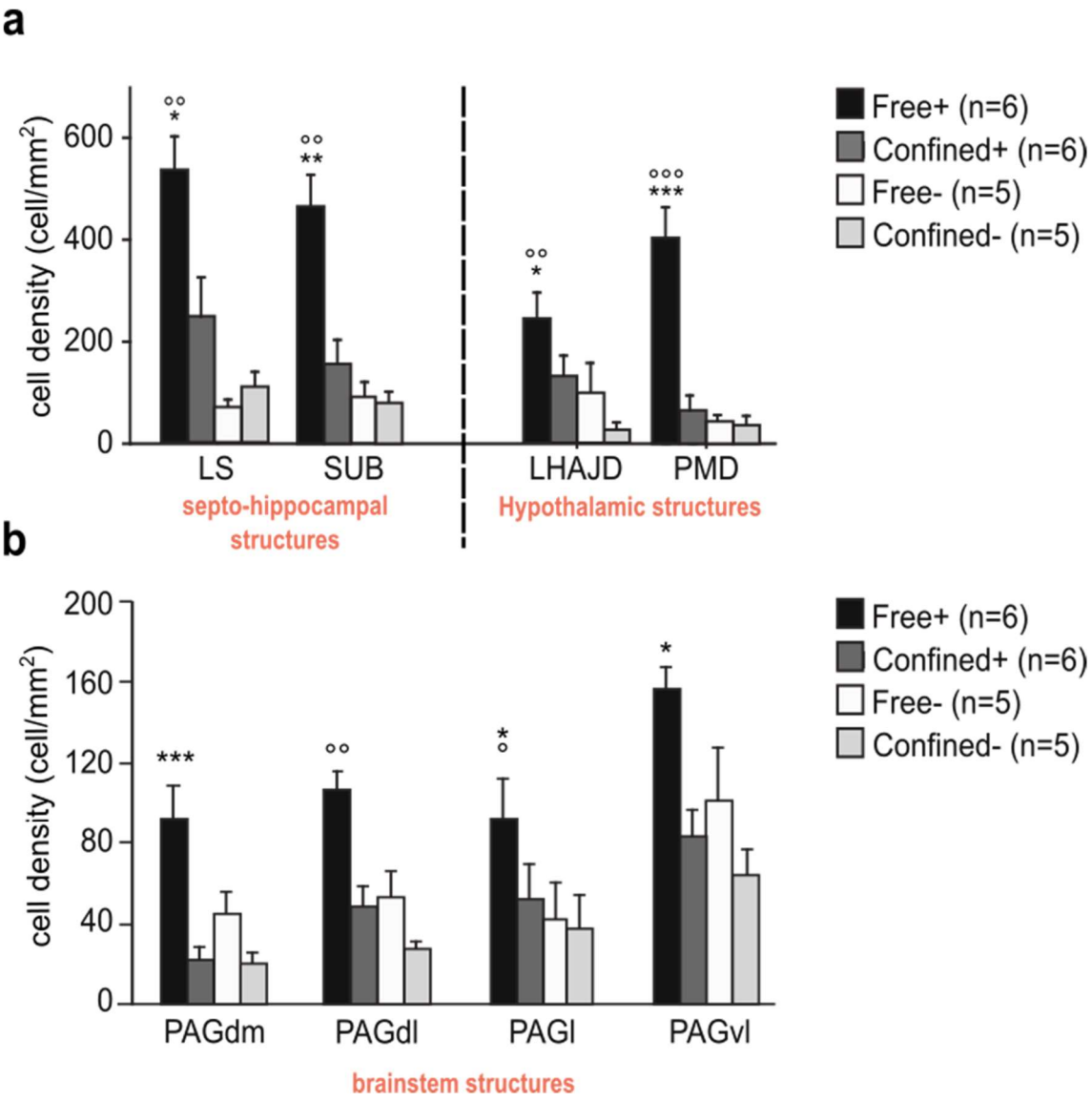

Figure 17 Density of Fos-labeled cells in selected brain regions during exposure to the context. The structures selected showed an up-regulation in Fos expression in the **Free+** group compared to the **Confined+** group and controls. **a.** The **Free+** group showed Fos upregulation in septohippocampal structures: **LS**: Lateral Septum, **SUBv**: ventral part of the Subiculum; and in hypothalamic structures: **LHAJD**: lateral hypothalamic area juxtadorsomedial region, **PMD**: dorsal premammillary nucleus. **b.** The **Free+** group showed Fos upregulation in brainstem structures **PAGdm:** periaqueductal gray, dorsomedial part; **PAGdl:** periaqueductal gray, dorsolateral part; **PAGl:** periaqueductal gray, lateral part; **PAGvl:** periaqueductal gray, ventrolateral part. Bonferroni Post Hoc test, after a Group effect on the ANOVA $p<0.0083$). When differs significantly from conditioned groups, *$p<0.05$; **$p<0.01$; ***$p<0.001$). When differs significantly from Control groups, °$p<0.05$; °°$p<0.01$; °°°$p<0.001$.

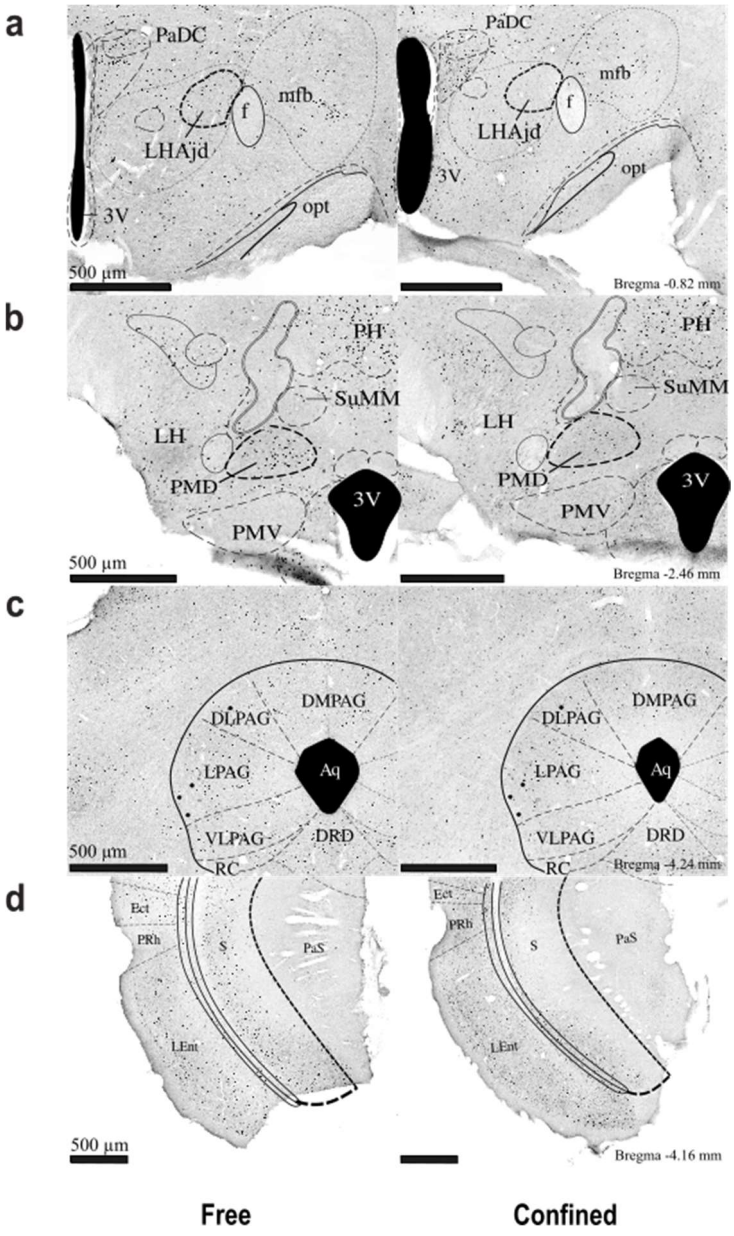

Figure 18 Photomicrographs of frontal Fos-stained sections illustrating the distribution of Fos-labeled cells in the LHAjd (a), PMD (b), PAG (c) and SUBv (d), comparing the Free+ (left column) and the Confined+ (right column) groups. Abbreviations: PaDC : paraventricular hypothalamic nucleus, dorsal cap; mfb : medial forebrain bundle; opt : olivary pretectal nucleus; LH: lateral hypothalamic area; Gem: gemini hypothalamic nucleus; PH: posterior hypothalamic area; SuMM: supramammillary nucleus, medial part; PMD: premammillary nucleus, dorsal part; PMV: premammillary nucleus, ventral part; DRD: dorsal raphe nucleus, dorsal part; PaS: parasubiculum ; Ect: ectorhinal cortex; PRh: perirhinal cortex; Lent: lateral entorhinal cortex 3V: third ventricle; f : fornix; LHAjd–lateral hypothalamic area juxtadorsomedial region; dlPAG – periaqueductal gray, dorsolateral part; dmPAG - periaqueductal gray, dorsomedial part; lPAG periaqueductal gray, lateral part; vlPAG – periaqueductal gray, ventrolateral part; PMD - dorsal premammillary nucleus; S: subiculum.

The overall data illustrate the idea that the fear conditioned animals which were able to avoid the conditioning chamber presented increased Fos expression in the circuit formed by the ventral subiculum, the juxtadorsomedial lateral hypothalamic area, 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.

III. Roles of the PMD on the non-instrumental contextual avoidance: Pharmacogenetic manipulation

After validating the recruitment of a specific pathway in our passive avoidance paradigm, we targeted the PMD, a nucleus of the pathway that, according to our hypothesis, is a key structure of the circuit. That is why we aimed at inactivating the PMD using pharmacogenetic tools. To do so we applied a CRE-dependent inhibitory DREADD virus (HM4D (Gi)) in the PMD in order to inactivate temporarily the structure with CNO during context exposure (Figure 19).

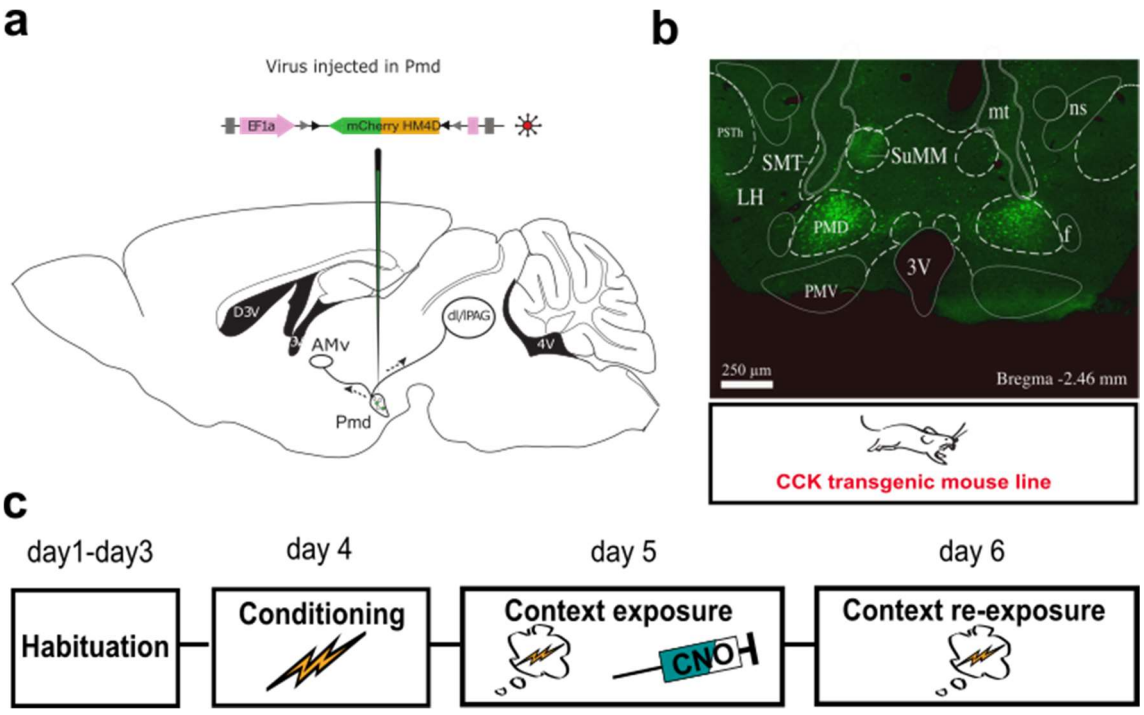

Figure 19 The inhibition of the PMD has an effect on the expression and the reconsolidation of passive avoidance a. Injection sites and viral strategy. Injection of a cre dependent virus expressing the DREADD inhibitory promoter. **b.** Photograph of the fluorescence of the DREADD infected cells of the PMD. The virus will recombine in the CCK positive cells of the PMD that express cre-recombinase in CCK-CRE-IRES mouse line. **c.** Behavioral protocol to test the PMD's role on the passive contextual avoidance. On **day 5** both groups (DREADD and GFP) were injected with CNO (300 µL intraperitoneal) 30 minutes before exposure to the context. On **day 6**, animals were placed again in the apparatus, without pharmacological treatment.

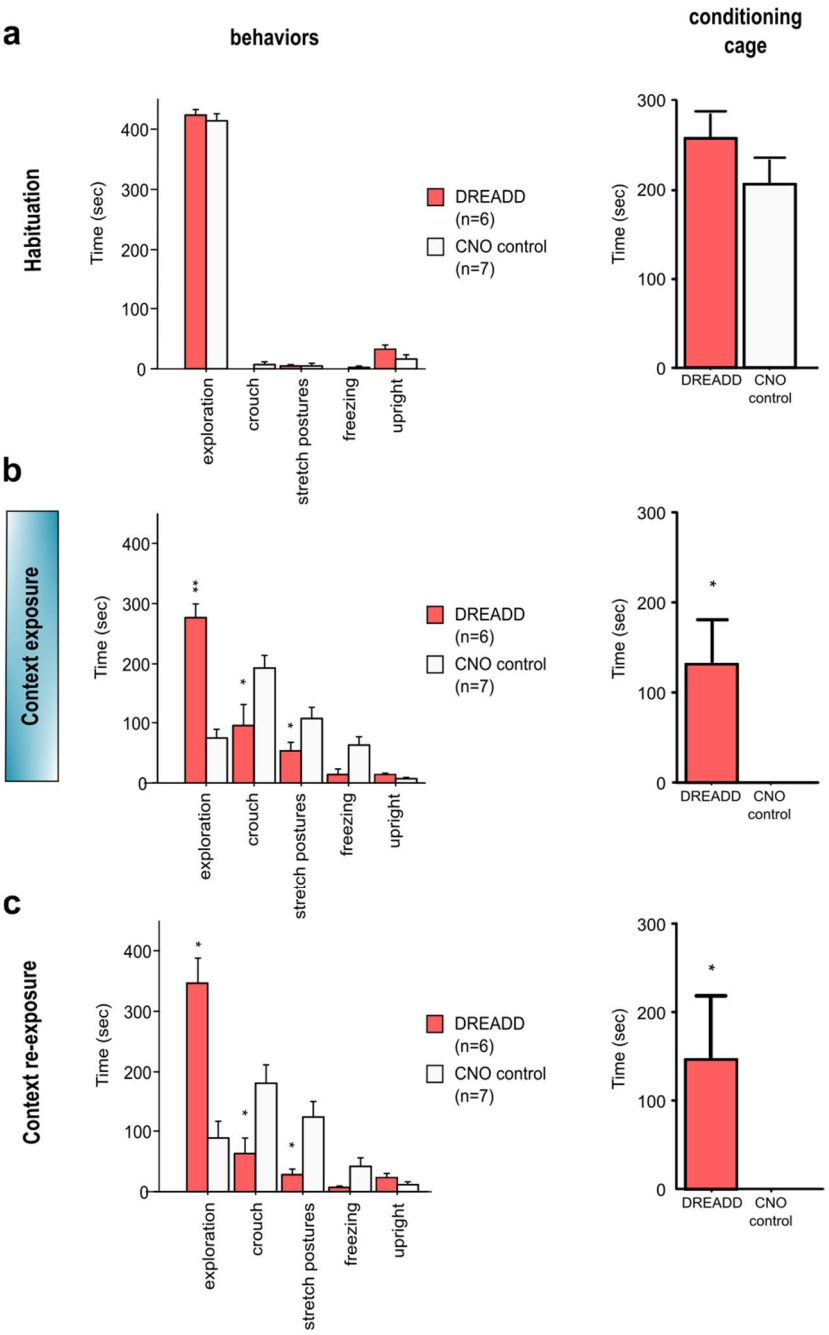

Figure 20 The inhibition of the PMD has an effect on the expression and the reconsolidation of passive avoidance. Behavioral and spatiotemporal analysis in the contextual avoidance apparatus during habituation **(a)**, first exposure to context **(b)** and re-exposure **(c)**. **a.** No behavior difference between the groups during **habituation**: group*behavior (two-way ANOVA $F_{(1,6)}$=0.864 p = 0.5259); and no time difference of time spent in the conditioning cage (unpaired t.test p=0.263). **b.** Behavior*group interaction difference during **context exposure** (two-way ANOVA F(1,6)=12,406 p <0.001); group effect on **exploration** p<0.01, **crouch sniff** p<0.05, and **stretch postures** p<0.05; and the **conditioning cage** (t-test p=0.0168). **c.** During **context re-exposure** there is also a difference between groups for the behavioral measurements (two-way ANOVA $F_{(1,6)}$= 16.557 p <0.001); group effect on **exploration**, **crouch sniff** and **stretch postures** p<0.05). The DREADD animals entered the **conditioning cage** (t-test p=0.0369).

On the last day of habituation (day 3), the behavior analysis show no difference between the DREADD and control group, as well as the time spent in the conditioning cage (Figure 20a.). On the context exposure day, there are significant differences between the DREADD and control groups in the behavioral expression and the time spent in the conditioning cage (Figure 20b.). In fact, the DREADD group drastically decreased risk assessment behaviors, like crouch sniffing and stretch postures, they also entered the conditioning cage, showing a loss of fearful recall of the conditioning day. Interestingly, during context re-exposure, we also found significant differences between the groups for the behavioral measurements, as well as the spatiotemporal measurements (Figure 20c.). Again, there was a significant decrease in risk assessment behaviors like stretch postures and crouch sniffing (Figure 20c. left panel). Yet again, while the control group wouldn't enter the conditioning cage, the DREADD group entered fearlessly the space, suggesting a impairment in memory reconsolidation of the context cues (Figure 20c. right panel).

IV. Behavioral and virus controls

Virus control

As described earlier in the ("Experimental protocols" section), all cre dependent viruses that we injected during our experiments infected CCK positive cells in CCK-IRES-CRE transgenic animals. In the posterior hypothalamus, these CCK positive cells are present exclusively in the PMD, and the rest of the mammillary bodies (ML, MM, MMn). Unfortunately, the injections targeting the PMD would also infect partially the mammillary bodies. According to the literature, cytotoxic lesions of the mammillary bodies as no effect on fear expression and recall during cat exposure in rats (Cezario et al., 2008). Acknowledging this information, we analysed the activation pattern of this region during passive avoidance, to make sure that the nuclei had no importance in our study as well.

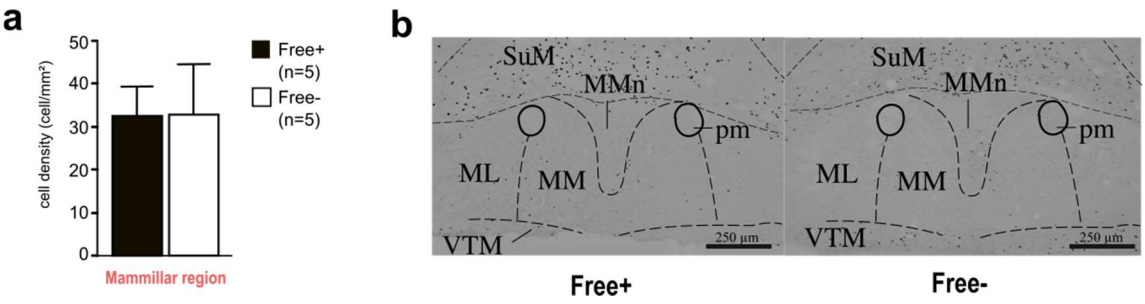

Figure 21 The mammillar nuclei present no activity during passive avoidance. a. cell density counting (cell/mm²) of fos immunostaining in the mammillar nuclei (ML, MM). There is no difference of Fos staining between the **Free+** and the **Free-** groups **:** t-test *p*=0.9647). **b.** Photomicrograph of a fos immunostaining of the mammillar region in a free+ animal (**left panel**), and its control (**right panel**). Abbreviations: : **MMn**: median mammillary nucleus, **ML**: medial mammillary nucleus, lateral part, **MM**: medial mammillary nucleus, medial part; **pm**: mammillary peduncle**; SuM**: supramammillary nucleus; **VTM**: ventral tuberomammillary nucleus.

When observing the Fos expression in the ML and the MM in the **Free+** and the **Free-** groups, there is only a very weak Fos-positive-cell density in the mammillar region (~32cell/mm²). Furthermore, the animals placed in free condition during context exposure (**Free+**) showed no difference of cell activation with the control (**Free-**) (Figure 21). These data comfort us in our hypothesis that the ML and MM are not implicated in passive avoidance.

Behavioral control

We then wanted to test whether the conditioning protocol would create generalisation. In order to do so we tested the risk of generalisation using a discrimination protocol. The animals would be presented to a neutral Open Field before entering the conditioning apparatus. We then observed the ratio of fearful versus fearless behaviors along the different sessions, in the two contexts (**Figure 22a.**, *see* **Experimental protocols**).

Our results illustrate that the behavior of the animals during the Open field exposure is not altered after the conditioning day, as there is no difference of ratio between the three days (**Figure 22b.**). Regarding the conditioning apparatus exposure, as expected, the post conditioning ratio of fearful vs. fearless behaviors drastically increases (**Figure 22c.**). These data suggest that the animals discriminate between the two contexts, and that only the conditioning apparatus becomes aversive after the conditioning day.

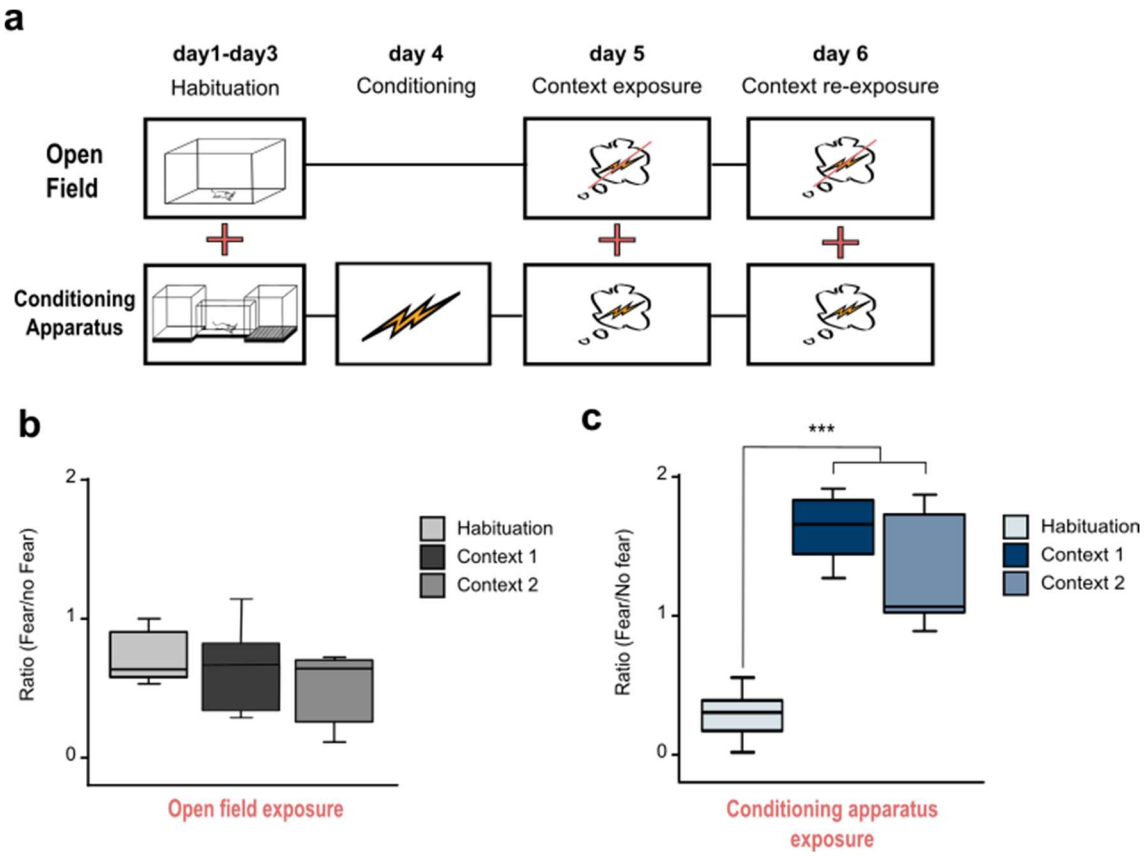

Figure 22 During Open field exposure, the fearful vs fearless behaviors ratio is not altered after the conditioning day, but the Conditioning apparatus exposure is. **a.** Contextual discrimination protocol. The animals were exposed to an open field 5 min before exposition to the conditioning apparatus during habituation days, exposure and re-exposure. **b.** Effect of the conditioning day on the ratio of fearful behaviors versus fearless behaviors, in the open field, n=7, ANOVA ($F_{(2,18)}$=0.263 $p < 0.77$). **c.** Effect of the conditioning day on the ratio of fearful behaviors versus fearless behaviors, in the conditioning apparatus, n=7, ANOVA ($F_{(2,18)}$=28.069 $p < 0.001$); Fisher'PLSD post hoc test (cont1 vs. cont2) p=0.1843; (Hab vs. cont1) p<0.001; (Hab vs. cont1) p<0.001.

Overall, after showing that the viral infection of the mammillar bodies does not disturb the behavior response as it is not involved in passive avoidance, and that the test does not create generalisation, it can be proposed that inactivation of PMD affects contextual passive avoidance. It also greatly weakens the process of reconsolidation of the aversive context.

V. role of the PMD projections in both the expression and reconsolidation of passive avoidance

Our next goal was to understand the role the terminal fields of the PMD, which are the dlPAG and AMv (Figure 23). These to structures are densely targeted by the PMD, and using optogenetic terminal inhibition we are going to examine their potential roles in the expression and reconsolidation of contextual avoidance.

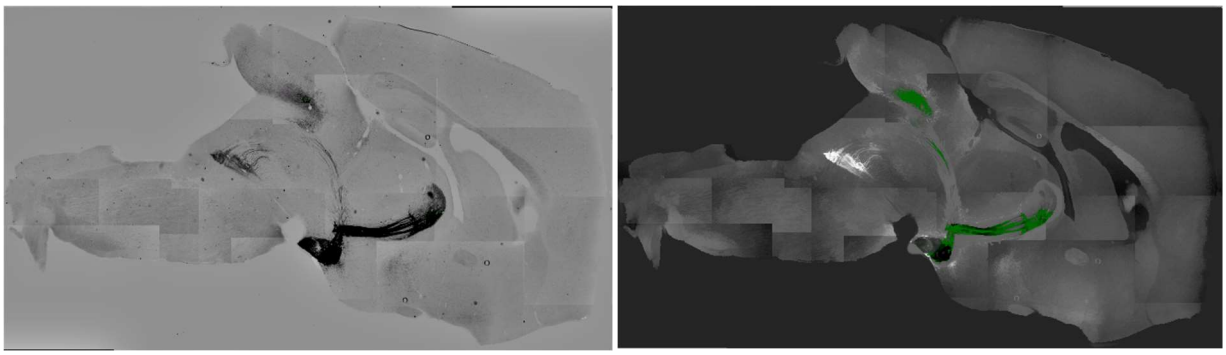

Figure 23 Sagittal views of the PMD projections in the ventral part of the anterior thalamus and the dorsal part of the PAG. **left panel**, projections of virus infected cells in the PMD and Mammillary bodies. **Right panel**, green coloration representation of the PMD projections only to the PAG (left direction), and the AMv, (right direction).

a. Optogenetic Manipulation on dlPAG terminals

We first inhibited the terminals of the PMD in the dlPAG using Halorodpsin virus inhibition during the context exposure (Figure 24).

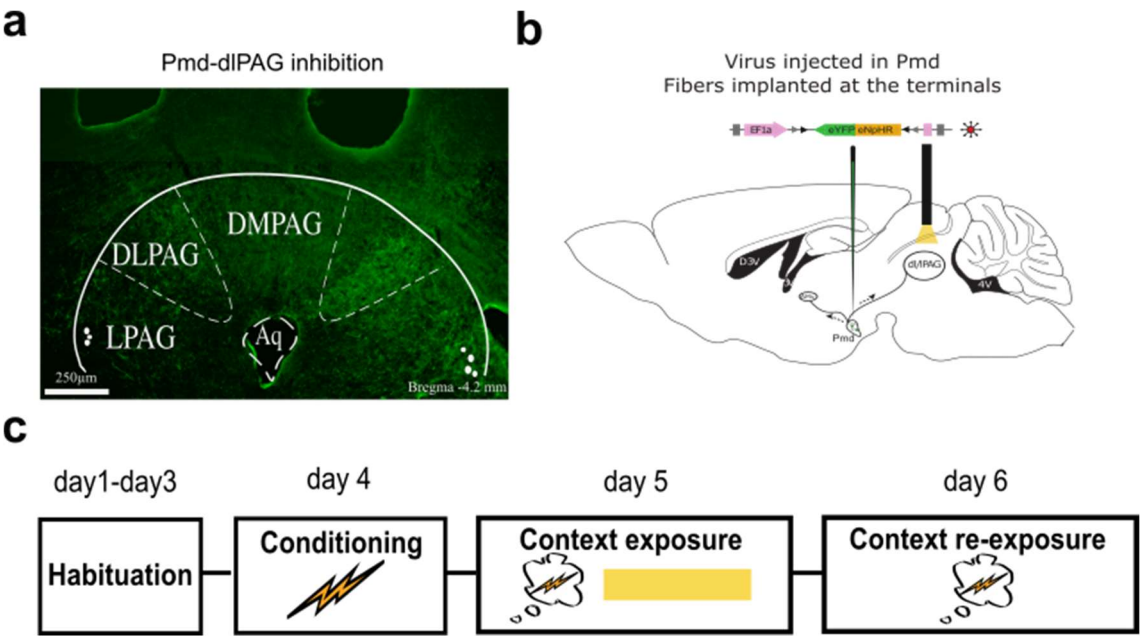

Figure 24 Optogenetic dPAG terminal inhibition strategy **a.** Photomicrographs of the PMD's terminal fields in the dlPAG . **b.** scheme of the viral and optogenetic inhibition strategy. CCK-IRES-Cre transgenic male mouse that received CRE dependent virus for the expression of Halorodopsin and the reporter YFP (rAAV9/CAG-Flex-eNpHR-YFP) in the PMD. Optical stimulation of the PMD terminals in the dlPAG. **c.** Behavioral protocol of the role of the PMD to dlPAG projections in passive avoidance. on **day 5**, both groups (Halorodopsin and GFP) were continuously stimulated with yellow light (wavelength of 589nm) during 8 minutes, while entering the conditioning apparatus. On **day 6**, the animals were re exposed to the same context but were not optically stimulated.

On the third day of habituation, the two groups presented no differences of behaviors, nor spatiotemporal distribution (Figure 25a.). However, the day of exposure to the aversive context (day 5), the Halo group significantly reduced risk assessment behaviors expression compared to the control GFP group. In fact, the inhibited animals expressed more exploration behavior, and less crouch sniffing and stretch postures (Figure 25b.left panel). They also entered the conditioning cage, with an average of 50 sec during the 8min of exposure (Figure 25b.right panel), whereas the control group never entered the cage.

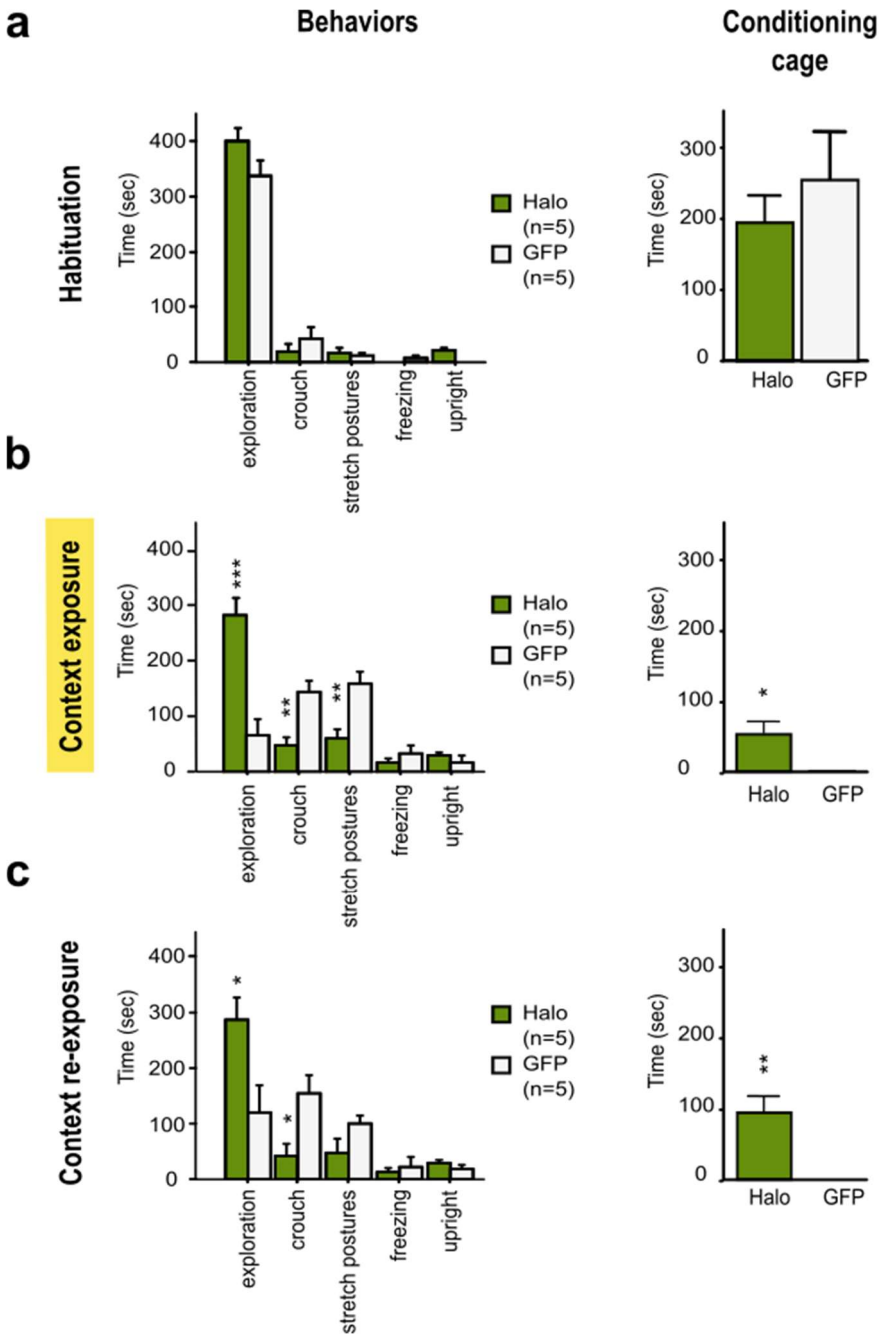

Figure 25 **Behavioral and spatiotemporal analysis in the contextual avoidance apparatus during habituation, first exposure to context and re-exposure**. **a. left panel** There is no difference during habituation behavior*group interaction two-way ANOVA $F_{(1,6)}$=2.087, p=0.0699. **right panel** There is no difference of time spent in the conditioning cage between the two groups. **b. left panel** the two groups show behavior differences during **context exposure**. Group*behavior interaction two-way ANOVA $F_{(1,6)}$=19.141, p<0.0001. T-test group difference in **exploration** (p=0.0002); **crouch** (p=0.0034); **stretch postures** (p=0.0052). **right panel** the Halo group entered the conditioning cage, but not the GFP group. (t-test, p=0.0227) **c. left panel** the two groups show behavior differences during **context re-exposure**. Group*behavior interaction two-way ANOVA $F_{(1,6)}$=5.635, p<0.0001. t-test group differences in **exploration** (p=0.0242); **crouch** (p=0.0159). **right panel** Halo group entered the conditioning cage, but not the GFP group (t-test p=0.0038).

On the 6th day, the animals where exposed again in the same aversive context, they also decreased in risk assessment responses. They expressed more exploration, and less crouch than the control group (Figure 25c.left panel). However, the stretch postures expression is not different from the controls (Figure 25c.left panel). They also entered the conditioning cage on the second day, which was still not the case for the control group (Figure 25c.right panel).

VI. Optogenetic Manipulation on Thalamic terminals

In a second part, we inhibited the terminals of the PMD in the AMv using Halorodpsin virus inhibition during the context exposure (Figure 26).

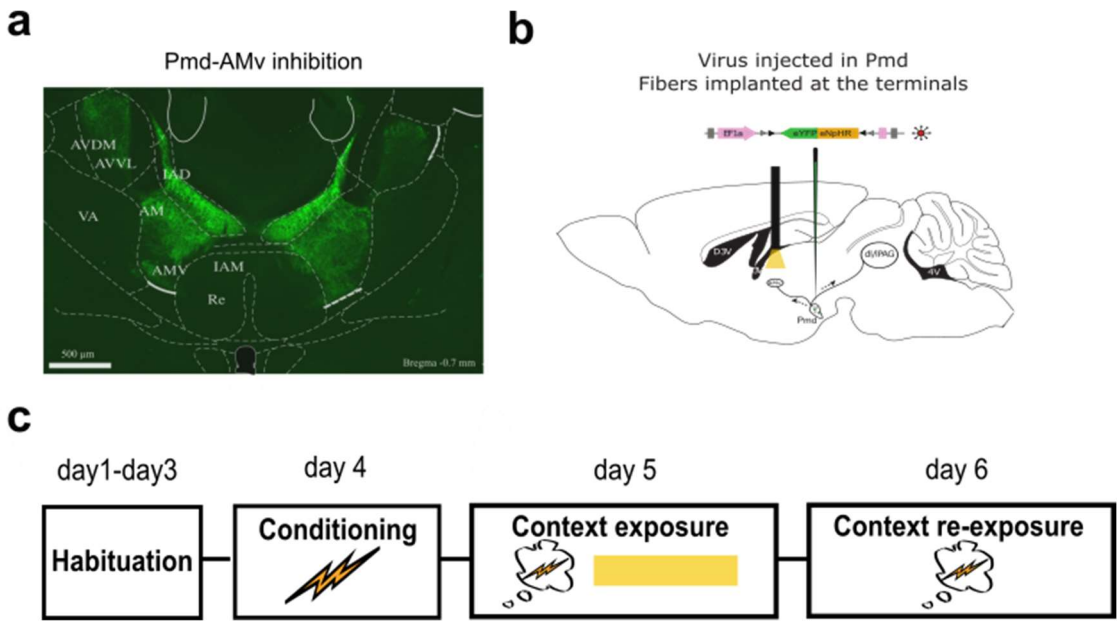

Figure 26 Optogenetic Thalamus terminal inhibition strategy. strategy **a.** Photomicrographs of the PMD's terminal fields in the AMv. **b.** scheme of the viral and optogenetic inhibition strategy. CCK-IRES-Cre transgenic male mouse that received CRE dependent virus for the expression of Halorodopsin and the reporter YFP (rAAV9/CAG-Flex-eNpHR-YFP) in the PMD. Optical stimulation of the PMD terminals in the AMv. **c.** Behavioral protocol of the role of the PMD to AMv projections in passive avoidance. on **day 5**, both groups (Halorodopsin and GFP) were continuously stimulated with yellow light (wavelength of 589nm) during 8 minutes, while entering the conditioning apparatus. On **day 6**, the animals were re exposed to the same context but were not optically stimulated.

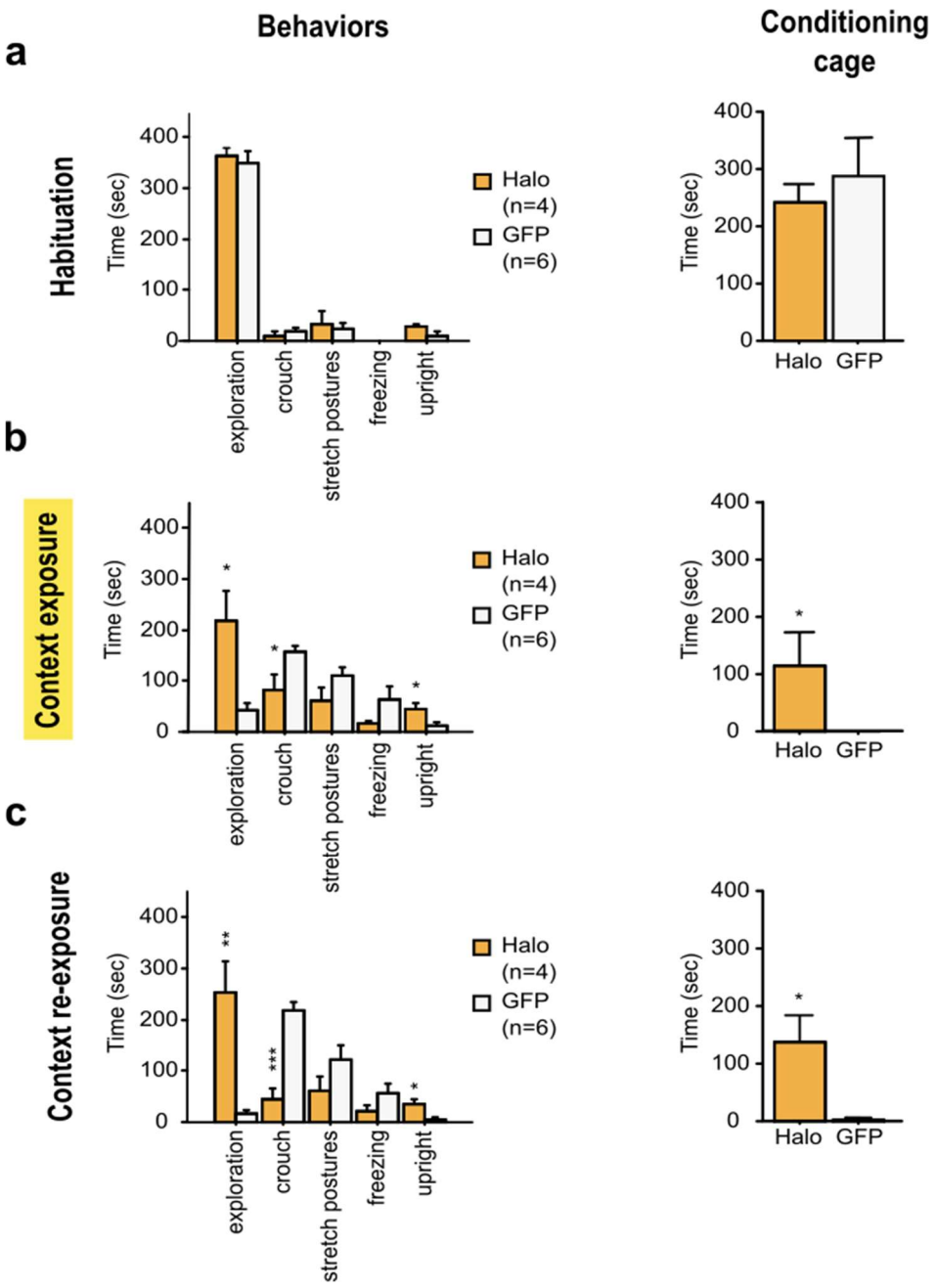

Figure 27 Behavioral and spatiotemporal analysis in the contextual avoidance apparatus during habituation, first exposure to context and re-exposure. **a. left panel** There is no difference during habituation behavior*group interaction two-way ANOVA $F_{(1,6)}$=0.351, p=0.9052. **right panel** There is no difference of time spent in the conditioning cage between the two groups. **b.** left panel the two groups show behavior differences during **context exposure**. Group*behavior interaction two-way ANOVA $F_{(1,6)}$=7.525, p<0.0001. t-test group difference in **exploration** (p=0.0121); **crouch** (p=0.0391); upright (p=0.0337). **right panel** the Halo group entered the conditioning cage, but not the GFP group. (t-test, p=0.0643) **c. left panel** the two groups show behavior differences during **context re-exposure**. Group*behavior interaction two-way ANOVA $F_{(1,6)}$=14.681, p<0.0001. t-test group differences in **exploration** (p=0.0039); **crouch** (p=0.0003); **upright** (p=0.0115). **right panel** Halo group entered the conditioning cage, but not the GFP group (t-test p=0.0270).

On the third day of habituation, the two groups present no differences of behaviors, nor spatiotemporal distribution (Figure 27a.). The results showed a difference in behaviors between the Halo group and its control during context exposure. The Halo group expressed more exploration and upright behaviors, and less crouch sniffing compared to the control (Figure 27b. left panel). Interestingly the amount of stretch postures expression does not differ with the controls. The Halo group also entered the conditioning cage, with an average of 50 seconds during the 8 minutes of exposure whereas the control group did not enter the compartment (Figure 27b. right panel). These results suggest that the inhibition of the AMv terminals partially impairs passive avoidance behaviors during context exposure.

On the re-exposure day, the Halo group also decreased in risk assessment responses compared to the control group (Figure 27c. left panel). They expressed more exploration and upright behaviors, and less crouch sniffing than the control group. However, the stretch postures expression is not different from the controls. When re exposed to the aversive context, the Halo group also entered the conditioning cage, which was still not the case of the control group (Figure 27c. right panel). According to these data the yellow light stimulation also impacted the re-exposure day, suggesting an effect on memory reconsolidation.

projections of the PMD to AMv and dlPAG are both involved in expression and reconsolidation of passive avoidance. However, even if the comparison with their controls shows a tendency of the dlPAG inhibition to disrupt more the expression of the behaviors, and the inhibition of the AMv has more effect on the re-exposure day which indicates a more important role in memory reconsolidation, the two groups don't differ between them (data not shown). Knowing the latter, no conclusion can be made about their specific implication differences in passive avoidance expression and reconsolidation.

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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