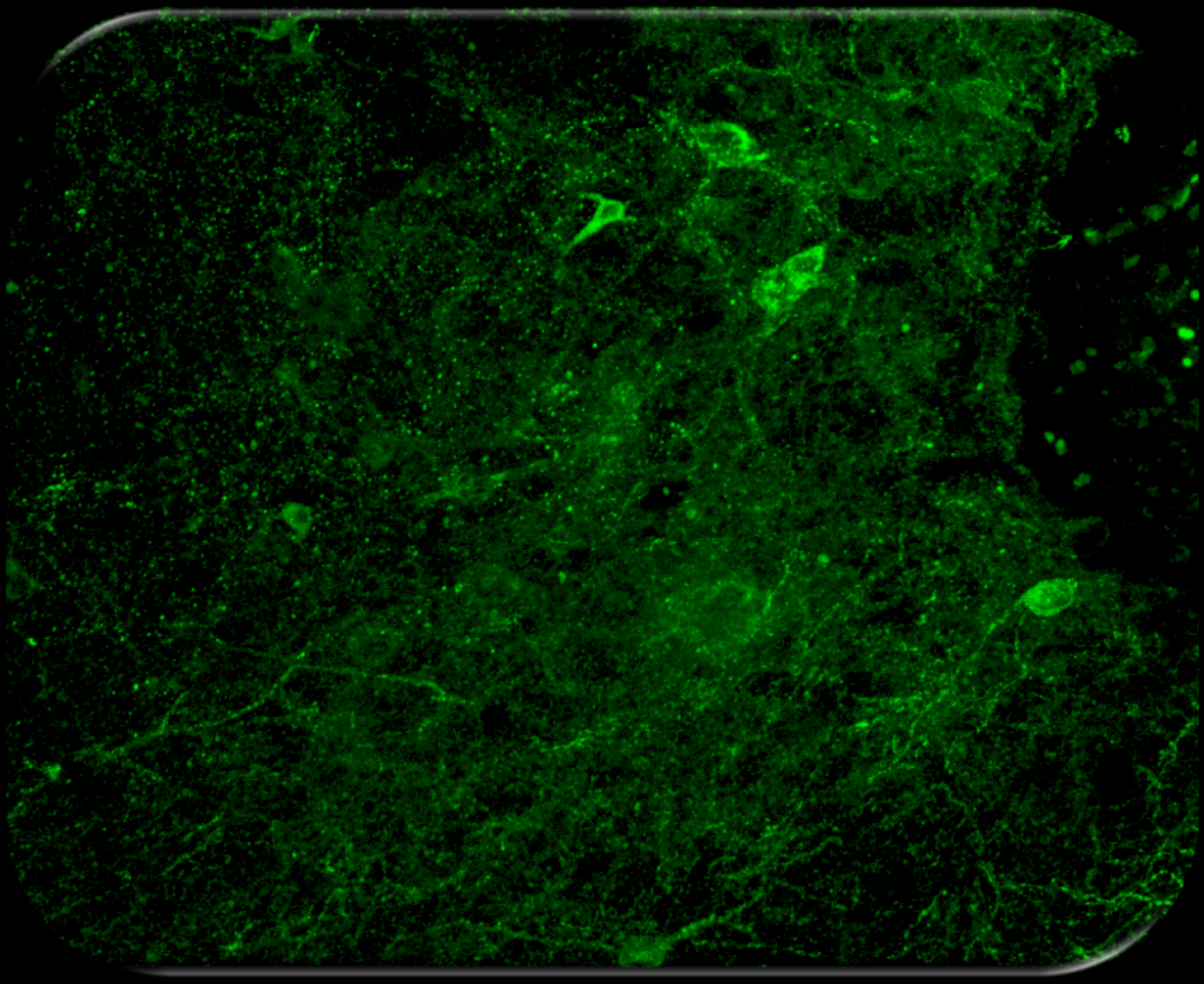
Auditory Cues in Social Transmission of Fear

Ana I. P. G. Pereira



Dissertation presented to obtain the Ph.D degree in Neuroscience

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
September,
2015

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Knowledge Creation

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To my grandfather

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Acknowledgments

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ABBREVIATION LIST

AFC	auditory fear conditioning
AOS	acessory olfactory system
BL	basolateral amygdala
BM	basomedial amygdala
CD	conditioned demonstrator
CeA	central amygdala
CeL	lateral portion of the central amygdala
CeM	medial portion of the central amygdala
ChR2	Channelrhodopsin2
CoA	cortical amygdala
CR	conditioned response
CS	conditioned stimulus
EO	experienced observer
ICC	inferior culliculus
IEG	immediate early gene
LA	lateral amygdala
MeA	medial amygdala
MGB	medial geniculate body
MGm	medial division of the medial geniculate body
MOS	main olfactory system
ND	naïve demonstrator
NO	naïve observer
PAG	periaqueductal gray
PIN	posterior intralaminar thalamic nucleus
SC	superior culliculus

SCR	skin conductance response
SI	social interaction
SSDR	species-specific defense responses
STF	social transmission of fear
UR	unconditioned response
US	unconditioned stimulus
USVs	ultrasonic vocalizations
VMH	ventralmedial hypothalamus

RESUMO

Quando um individuo detecta uma ameaça no seu ambiente, são desencadeadas alterações tanto a nível fisiológico como comportamental. Estas alterações podem ser detectadas por outros indivíduos que se encontram nas proximidades, alterando o comportamentos deste últimos. Esta transferência de informação relativa a um perigo iminente é denominada Transmissão Social de Medo. A utilização de informação social que sinaliza a presença de uma ameaça pode ter benefícios a curto prazo (como seja evitar um perigo eminente), ou estar na base da aprendizagem social sobre novas ameaças. Apesar da prevalência deste fenómeno no reino animal, bem como a sua importância para a sobrevivência, pouco se sabe sobre os mecanismos neuronais subjacentes.

Com o objectivo de adereçar esta questão, desenvolvemos um paradigma comportamental que permitisse estudar transmissão social de medo em contexto laboratorial, utilizando ratos como animal modelo. Para tal, no nosso paradigma utilizamos pares de ratos em que um deles é designado de demonstrador e o outro de observador. O teste de Interação Social é realizado numa caixa de acrílico dividida ao meio por uma partição que separa os dois ratos. Esta partição permite que os ratos se vejam, oiçam, cheirem e toquem. Durante a interação social é apresentado um som (estímulo condicionado) ao qual o demonstrador foi adversamente condicionado no dia anterior. A apresentação do estímulo condicionado desencadeia imobilidade nos demonstradores, uma prevalente resposta de medo. Confirmando resultados prévios, verificámos que as respostas de medo dos demonstradores desencadeiam imobilidade nos observadores, caso estes últimos tenham tido uma experiência prévia com choques. Ao investigar quais as modalidades

sensoriais que suportam a transmissão de medo, demonstrámos que os observadores não necessitam de contacto, informação visual ou auditiva conferida por gritos de alarme para expressarem uma resposta de medo desencadeada pela imobilidade dos seus conspecificos. Utilizam no entanto informação auditiva que sinaliza a transição súbita de movimento para imobilidade. Durante a fase inicial da interação social, os ratos movem-se na caixa produzindo sons característicos do seu movimento. Estes sons diminuem drasticamente quando o demonstrador fica imóvel. Foi assim hipotetizado que esta transição do som do movimento para silêncio é necessária para a imobilidade dos observadores. De forma a testar esta hipótese, reproduzimos o som de um rato a mover-se durante a fase da interação social em que ambos os ratos estão imóveis, abolindo o silêncio. Verificámos que a reprodução deste som aboliu a imobilidade dos observadores, e ainda que a sua terminação desencadeou de novo imobilidade nestes últimos.

De forma a testar se a cessação do som do movimento é suficiente para desencadear imobilidade, realizámos um outro conjunto de experiências em que colocámos ratos com experiência previa com choques na mesma caixa de interação social, mas desta vez sozinhos. Durante a sessão de teste o mesmo som proveniente do movimento de um rato foi reproduzido dentro da caixa de forma continua, com exceção de dois períodos de um minuto de silêncio. Os sujeitos estiveram mais tempo imóveis durante esses períodos de silêncio em comparação com os períodos de mesmo duração anteriores à cessação do som. Estas experiências confirmam que a cessação do som do movimento é necessária e suficiente na transmissão social de medo.

Grande parte do que se sabe relativamente à expressão de comportamentos defensivos desencadeados por sons resulta de estudos de

Condicionamento Auditivo de Medo. Estes estudos demonstraram que a Amígdala lateral é necessária para a aprendizagem, armazenamento e expressão de medo em resposta a estímulos sonoros. Porem, na maior parte destes estudos a resposta de medo é desencadeada pela apresentação de um som, enquanto que no nosso caso é decorrente da sua cessação. De forma a investigar quais os mecanismos neuronais subjacentes à expressão de medo desencadeado pela súbita cessação do som do movimento, começámos por averiguar se a atividade na amígdala lateral também é importante para a expressão de medo desencadeada pelo nosso estimulo.

Para tal, recorremos a uma manipulação optogenética para inibir a Amígdala lateral especificamente durante o intervalo de silêncio introduzido no som do movimento. Nesta experiência utilizámos 2 grupos, ArchT (que expressa a bomba de protões ArchT na Amígdala lateral permitindo a sua inibição) e Controlo. Comparando a percentagem de tempo que os ratos estiveram imóveis durante o período de silêncio e o período antecedente a este no grupo ArchT, não foram encontradas diferenças estatisticamente significativas. Pelo contrário, os animais controlos estiveram imóveis significativamente mais tempo durante o silêncio comparado com o período que o precede. Verificou-se ainda uma diferença significativa na imobilidade entre os animais do grupo ArchT e Controlo durante o silêncio, mostrando que atividade neuronal na Amígdala lateral é importante para a expressão de respostas de medo desencadeadas pela cessação do som do movimento.

De forma a averiguar quais as vias neuronais envolvidas na detecção da súbita terminação do som, analisámos a expressão de *c-fos* (um gene cuja expressão se correlaciona com a atividade neuronal) em diferentes núcleos do Corpo Geniculado Medial do tálamo auditivo. Estudos prévios de electrofisiologia realizados em animais anestesiados reportaram a existência

de células que respondem à terminação de diferentes sons em vários subnúcleos desta região. Estudos anatómicos revelaram ainda que existem projeções diretas destes subnúcleos para a Amígdala lateral.

A análise da expressão de *c-fos* revelou um aumento significativo no numero de células marcadas pela proteína *c-fos* no núcleo dorsal do Corpo Geniculado Medial em animais expostos ao som do movimento com intervalos de silêncio, quando comparado com animais sujeitos a este som de forma contínua. Este aumento foi particularmente marcado na zona mais posterior deste subnúcleo. Uma vez que esta região tem projeções diretas para a Amígdala lateral (confirmadas no presente estudo), sugerimos que a ativação de células neste subnúcleo, desencadeada pela cessação do som do movimento, leva à ativação de células pos-sinapticas na Amígdala lateral desencadeando imobilidade.

Em resumo, durante o decurso deste trabalho desenvolvemos um paradigma experimental que permite estudar transmissão social de medo em ratos no contexto laboratorial. Identificámos um estimulo auditivo que é tanto necessário como suficiente para desencadear respostas de defesa em observadores. Descobrimos ainda que é necessário que a Amígdala lateral esteja ativa para que haja uma resposta comportamental a este estímulo, e que este é provavelmente sinalizado pelo subnúcleo dorsal do Corpo Geniculado Medial do talamo auditivo. Com este estudo contribuímos assim para o conhecimento dos mecanismos neuronais subjacentes à transmissão social de medo bem como ao processamento de sons etologicamente relevantes no cérebro no contexto do medo.

ABSTRACT

When an animal faces a threat, both behavioral and physiological changes occur that promote the avoidance of the menace. Individuals in the surroundings of the fearful animal (both con and heterospecifics) may detect some of these changes, that become cues that signal an impending danger. The detection of such cues can therefore trigger defense behaviors in observers, in a phenomenon called Social Transmission of Fear.

The use of social information to signal danger can have both immediate benefits like the avoidance of the menace, or underlie social learning about threats. Despite its prevalence and importance for survival, very little is known about the neuronal mechanism underlying it.

In an attempt to address this question, we developed a behavioral paradigm in the laboratory to study social transmission of fear using rats as an experimental model. In our experiments, a pair of cage-mate rats (one assigned to be the demonstrator and the other the observer) interacted in a two-partition chamber, which allowed rats to see, hear, smell and touch each other. During the social interaction test we presented a tone cue (conditioned stimulus), to which the demonstrator rat had previously been conditioned (conditioned demonstrator). The presentation of the conditioned stimulus triggered freezing, characterized by complete immobility, in demonstrators. Confirming previous studies, we found that observer rats freeze while witnessing the demonstrator display fear responses, provided they had prior experience with an aversive footshock. By systematically probing for the sensory cues that trigger transmission of fear, we show that observer rats do not rely on contact with the demonstrator, visual cues or alarm calls to detect fear. Instead, they use changes in auditory cues in the environment that are

likely to signal the sudden transition from motion to immobility. During the baseline period rats move around in the social interaction chamber producing rustling sounds, which decreased dramatically when the demonstrator rat started freezing. We then hypothesized that the transition from the sound of movement to silence is necessary to trigger freezing in observers. In order to test this hypothesis we played back the sound of a rat moving while both rats were immobile, disrupting silence. We found that the playback of the movement-evoked sound disrupted freezing by observers and that freezing resumed immediately after the sound playback re-instated by silence.

In another set of experiments we placed experienced rats alone in the social interaction chamber. During the test session the same movement-evoked sound used in the previous experiment was played continuously, except for two one-minute periods of silence. Experienced rats significantly increased their levels of freezing during the periods of silence compared with baseline. These experiments confirm that the absence of movement-evoked sound is necessary and sufficient to induce fear in observer rats.

Most of what is known about acoustically driven defense behaviors was unrevealed by Auditory Fear Conditioning studies that demonstrated that the Lateral Amygdala is necessary for learning, storage and expression of defense behaviors triggered by sounds. However, most of these studies used artificial sounds like pure tones and the defense responses are triggered by the presentation of a sound. In order to investigate the neuronal pathways underlying fear expression triggered by the sudden cessation of the sound of movement, we investigated if activity in the Lateral Amygdala is necessary for fear expression triggered by this stimulus.

For this purpose, we optogenetically inhibited the Lateral Amygdala specifically during a silence gap introduced during the playback of the movement-evoked sound. For this experiment we had two groups: ArchT (expressing the ArchT proton pump in Lateral Amygdala which when stimulated by light allows neuronal inhibition) and Control (with fiber implants but no ArchT expression in Lateral Amygdala). We didn't found any significant increase in freezing between baseline and silence in the ArchT group. On the contrary, such an increase was found in the Control group. Moreover, there was a significant difference in percentage of freezing between the ArchT and the Control group during the period of silence, showing that activity in Lateral Amygdala is important for the display of defense responses triggered by the cessation of movement-evoked sounds.

In order to address the neuronal pathways involved in the detection of a sudden termination of a sound, we compared *c-fos* expression in different subnuclei of the auditory thalamus of animals exposed to the movement evoked sound interrupted by silence to that of animals exposed to continuous sound. Previous electrophysiology studies performed in anaesthetized rodents reported offset cells in several of the subnucleus of the auditory thalamus. Also, direct projections from this subnucleus to the Lateral Amygdala have been described. We found a significant increase in the number of *c-fos* expressing cells in the dorsal part of the Medial Geniculate Body of the thalamus in rats exposed to the silence gaps, being this increase more significant in the more posterior part of this subnucleus. Given the direct connections between cells in this nucleus and the Lateral Amygdala (also confirmed in the following study), we hypothesize that activation of cells in the dorsal part of the Medial Geniculate Body by the cessation of the movement-evoked sound drives activity in pos synaptic cells in Lateral Amygdala

triggering freezing.

Summing up, we developed a behavioral task that allows the study of social transmission of fear in rats under laboratory settings. We were able to isolate an auditory cue that is both sufficient and necessary to trigger defense behaviors in conspecifics. We found that activity in Lateral Amygdala is necessary for the display of freezing triggered by this auditory cue, and that the dorsal part of the Medial Geniculate Body of the auditory thalamus is a candidate structure in signaling the cessation of the movement evoked sound. Therefore, this study contributes to a better understanding of the neuronal mechanisms underlying transmission of fear as well as how ethologically relevant sounds are processed in the brain in the context of fear.

CHAPTER I – GENERAL INTRODUCTION

"*The most common mutual service in the higher animals is towarn one another of danger by means of the united senses of all.Every sportsman knows, as Dr. Jaeger remarks (7. "Die Darwin'sche Theorie", s.101.), how difficult it is to approach animals in a herd or troop. Wild horses and cattle do not, I believe, make any danger-signal; but the attitude of any of them who first discover an enemy warns the others"*

Charles Darwin in "*The Descent of Man"* (1871)

Chapter I - General Introduction

Although fear is an emotion that most likely has been felt by the majority of humans, its definition is far from being unanimously agreed upon. Fear can be defined as the conscious feeling of being afraid of an impending danger. However, behavioral neuroscientists have classically defined and studied "fear" and "fear responses" as the set of physiological and behavioral changes that occur in response to stimuli that signal a potential threat. The main debate arises from how related the mechanisms underlying the last are with the ones that generate the conscious feeling of being afraid (LeDoux JE 2014[1], Gross C 2012[2], Adolphs R 2013[3]). This debate is of particular importance when studying the feeling of fear in humans, and whether it can be extended to other animals. However, the study of the neuronal mechanisms underlying defense responses, the classically called fear system, is of major importance not only to understand the emotion of fear, but also the survival and adaptation of different species to their environment.

In the context of this thesis, the fear system will be approached based on the circuits which underlie the display of defense responses, including its behavior output as well as the physiologic and autonomic changes concomitant to it.

Defense responses can be triggered when an individual directly recognizes a threat. Fear can be expressed right from the first encounter with a threat or with a cue that signals it, being presumably innate (Veen T 2000[4], Du Y 2012[5], Goth A 2001[6], Gross C 2012[2]). Fear responses can also be learned; in this case a first encounter with the potential threat (or once again, a cue that signals it) does not trigger defense behaviors. However, after an association is made between it and an innately aversive stimulus, the next encounter with

such threat will lead to the display of fear. Several years of research in the fear system has led to extensive literature regarding both innate and learned fear behaviors triggered by the direct detection of a threat (for reviews see Gross C 2012[2], Adolphs R 2013[3], Herry C 2014[7]).

Importantly, information conveyed by others can also be used to detect threats in the environment. Social transmission of fear takes place when cues provided by an individual (demonstrator) trigger the display of defense responses in other individuals, either con- or heterospecifics (observers). These cues are the result of changes in physiological and behavioral responses of demonstrator individuals upon the direct detection of a threat. The responses of observers to such social cues can be either innate or learned (Hollen LI 2009[8], Enjin A 2013[9]). Although currently there are several known examples of transmission of fear in wild populations (Seyfarth RM 1980[10], Zuberbuhler K 2001[11], Ito R 2009[12], Wilson DR 2004[13], Ono M 2003[14], Hingee M 2009[15], Coleman SW 2008[16], Curio E 1978[17]), the mechanisms underlying it are still largely unknown. Only more recently have studies started being performed under laboratory settings (Masuda A 2009[18], Kim EJ 2010[19], Jeon D 2010[20], Sanders J 2013[21], Chen Q 2009[22], Atsak P 2011[23], Bruchey AK 2010[24], Olsson A 2007[25], Jones CE 2014[26], Church RM 1959[27]).

The work developed during the following thesis focused on social transmission of fear using rats as an experimental model, and attempted to unravel the neuronal mechanisms underlying it. Our main goals were:

1) To establish a behavioral paradigm for the study of social transmission of fear under laboratory settings;

2) To unravel the sensory cues underlying this behavior;

3) To investigate the brain regions involved in the response to sensory cues provided by others upon the perception of a threat;

As previously mentioned, very little is known about the neuronal mechanisms underlying social transmission of fear. The first part of the following introduction will thus focus on the characterization of defense behaviors and on the description of the neuronal pathways underlying both innate and learned fear responses when they are triggered by the direct recognition of the threat. A review of this literature may provide important insight into the potential brain regions and neuronal mechanisms underlying social transmission of fear.

The second part of our introduction will focus on the different examples of social transmission of fear in both wild and laboratory studies. We will review the sensory cues underlying this behavior in different species, with particular emphasis in rodents.

Part I - Neuronal Pathways Underlying Innate and Learned Defense Behaviors

I.I Defense responses

The fear system can be considered a survival circuit that responds to information about a potential threat with endocrine, autonomic and behavioral changes. The adopted behavior should be the one that enhances the prospect of avoiding or escaping the threat, minimizing injury, and increasing the likelihood of survival. The activation of this survival circuit may also lead to the formation of memories of this encounter, which may be useful in the future (Ledoux JE 2014[1]).

Defense behaviors are species specific, and presumably innate.

Bolles' (Bolles RC 1970[28]) outlined in his species-specific defense reactions (SSDR) theory that when an animal faces a threat, its behavioral repertoire becomes restricted to a set of prepackaged behaviors. Freezing, fight and flight are examples of these behaviors that are common to most animal species. According to this theory, SSDR can be rapidly acquired as avoidance responses in tasks where an animal has to perform an action in order to avoid an aversive stimulus. On the other hand, other behaviors of the animal's repertoire (e.g. grooming) may need extensive training or may never be learned as avoidance responses. Namely, a subject learns very fast to run away from an alley to avoid a shock because fleeing is part of his SSDR. However, extensive training is needed if the animal must bar press to avoid a shock, since this action is not in his defensive prepackaged repertoire.

The defense reaction displayed by the animal is however dependent on the context where it encounters the threat. The elicitation model suggested that

the biologically important features of the threat source and the characteristics of the environment determine the expression of a given defense behavior (Blanchard RJ 1969[29]). In particular, if an escape path is available, flight is usually the best response, however if no such escape exists, then freezing (characterized by complete immobility) is more advantageous (Blanchard RJ 1971[30]).

The above referenced studies also concluded that the environment might become associated with the threat itself (Blanchard RJ 1969[29]). Thus, although there are predefined defense behaviors, their expression is flexible depending on the characteristics of the surroundings. Moreover, it is influenced by the expectation of the threat that is provided by cues in the environment (Bolles RC 1976[31]). Subsequent studies by Fanselow and Lester lead to the development of the predatory eminence theory, which proposed that the prey's perceived likelihood of being caught by the predator is what determines the displayed defense behavior. This likelihood is influenced by the distance to, or the temporal probability of encountering the threat (e.g. if an animal is hidden, it would take longer for a predator to detect it than if it is in a open arena), and not only by the characteristics of the environment (Fanselow MS 1994[32]).

The above-mentioned models contributed to the contemporary view that there is a set of unlearned defense responses that are expressed when the subject is exposed to an intrinsically aversive stimulus independently of prior learning. However, they are not reflexes since they are flexible and modulated by the environment. The expression of one of these responses instead of the others is context dependent. Meaning, it is influenced by eliciting circumstances (e.g. if escape is available or not), the nature of the threat as well as the likelihood of encountering it.

Freezing, fight and flight are examples of such defense responses and have been reported as part of the coping strategies of almost all vertebrates (Mirza RS 2003[33], Oliveira R 2011[34], Gabrielsen GW 1985[35], Ellison K 2012[36], Blanchard RJ 1971[37], Mateo JM 1996[38], Roelofs K 2012[39]). These different coping strategies are generally categorized as active or passive. This categorization is based on changes in both the motor output and in the patterns of autonomic activity (Bandler R 2000[40], Bittencourt AS 2004[41]).

Following the classical categorization, fight and flight are considered active coping strategies characterized by increased motor activity, hypertension, tachycardia and non-opioid mediated analgesia. On the other hand, passive coping strategies are characterized by reduced somatomotor activity, sometimes hypotension and bradycardia, and opioid-mediated analgesia. Freezing, characterized by immobility, body tenseness, shallow breathing, exophtalamus and absence of sniffing, has been proposed as a passive coping strategy. However, it has also been proposed that freezing is a defense response during which the animal is highly attentive to the environment, being its classification as a passive response arguable.

I.II Neuronal Circuits of Innate and Learned Defense Behaviors

The aforementioned unlearned defense responses can be triggered by a variety of stimuli, and given the nature of the stimulus the resultant behavioral and/or physiological response can be further divided into innate or learned. Importantly, the same unlearned response (e.g. freezing) can be triggered by both an innately aversive stimulus such as a footshock (and in this case we are in the presence of an innate defense response), or by a learned cue that predicts an intrinsically aversive stimulus (like a tone previously paired with shock – learned defense response).

Innate defense responses are hence believed to result from the activation of developmentally programmed neuronal circuits, and the stimulus that triggers them is intrinsically threatening.

On the other hand, learned defense responses are triggered by a previously neutral cue that was associated with an innate aversive stimulus.

The Amygdala, located in the most ventral part of the mammalian brain, has been widely implicated in both learned and innate fear responses (reviewed in Gross C 2012[2], Pape H 2010[42], Maren S 2004[43], Herry C 2014[7]). Based on anatomy and cellular properties, it is subdivided into several subnuclei. The basolateral complex encompasses the lateral (LA), basolateral (BL) and basomedial (BM) subnuclei that receive most of the sensory inputs to amygdala. This complex is a cortex like structure and the most common cell types are multipolar, pyramidal-shaped or stellate projection neurons. These projection neurons mainly use the neurotransmitter glutamate, and they contribute to most of the projections to other amygdala nuclei and the rest of the brain.

One important projection site of this complex is the central nucleus of the amygdala (CeA) that in turn projects to brainstem nuclei responsible for the generation of different aspects of defense responses. It is believed that most neurons in the CeA are GABAergic, being that most of the projections arising from this nucleus are inhibitory (Pape H 2010[42]).

The medial (MeA) and cortical (CoA) amygdala are also important for the display of defense behaviors. They receive strong inputs from olfactory sensory areas, namely the main and accessory olfactory bulbs, processing information about predator odors and pheromones (Takahashi L 2014[44], Meredith M 2004[45], Root C 2014[46]). At the cellular level, The MeA is characterized by an

elevated number of glutamic acid decarboxylase (GAD) positive neurons (both interneurons and projection neurons (Keshavarzi S 2014[47])), whereas most cells in CoA are glutamatergic (Sah P 2003[48]).

Other brain regions like the Hypothalamus and the Periaqueductal Gray (PAG) are also part of the so called "fear circuit", being densely interconnected with the amygdala. The later is classically viewed as an output station downstream of amygdala, important for the coordination of the behavioral manifestations of the defense responses (freezing, fight or flight). In the further sections, we will review the contributions of these different structures and their subnuclei in the display of both innate and learned defense responses. The majority of these studies were performed in rodents.

a) Innate Defense Responses

Innate defense behaviors are mostly displayed in response to painful stimuli, predators, aggressive conspecifics or cues of different sensory modalities that signal the previous. Namely, a looming visual stimulus, which resembles an avian predator, triggers both freezing and escape responses in mice (Yilmaz M 2013[49], Shang C 2015[50], Wei P 2015[51]). It has also been shown that auditory stimuli like a broad band white noise (Xiong XR 2015[52]) or a train of 17-20KHz frequency sweeps (Mongeau R 2003[53]) delivered at high intensities, as well as a noxious somatosensory stimulus like a footshock, can equally trigger innate defense behaviors (Gross C 2012[2]).

The exposure of a rat to a cat significantly increases *c-fos* expression (an immediate early gene (IEG) normally used as a marker of neuronal activity) in the LA, posterior BM (pBM) and MeA. Lesions in these areas also decrease the expression of defense responses, in particular freezing. Notably, lesions of the

CeA, shown to be necessary for the expression of learned freezing, do not affect innate fear responses to cat exposure (Martinez RC 2011[54]). This suggests the existence of a pathway dedicated to the display of coping strategies towards predators. The posterior ventral MeA (pvMEA), a region that receives strong inputs from both the Main Olfactory System (MOS) and Accessory Olfactory System (AOS) seems to be particularly important in the response to cat odor. The LA and pBM, which are strongly interconnected and receive inputs from both auditory and visual sensory processing areas, are proposed to integrate non-olfactory predator-derived cues (Gross C 2012[2]). The pvMEA and the pBM project to the dorsalmedial part of the ventralmedial Hypothalamus (dmVMH), proposed to be part of a predator-responsive circuit in the hypothalamus. Pharmacogenetic inhibition of the dmVMH significantly decreases defense responses towards a predator in comparison with controls, but has no significant effect when the threat is an aggressive conspecific or a noxious footshock (Silva BA 2013[55]). This network targets the dorsolateral part of the PAG (dlPAG), and pharmacological inactivation of the dPAG (including the medial and lateral part) is sufficient to significantly reduce defense responses in mice to the presentation of a predator rat (Gross C 2012[2], Silva BA 2013[55]). These results suggest that the dlPAG is an important site for the orchestration of defense responses towards predators. One interesting aspect of the dlPAG is that it does not receive direct inputs from the spinal chord that receives cutaneous, deep somatic and visceral primary afferents that provide information about noxious stimulation. This suggests that this subnucleus might respond to stressors other than physical (Bandler R 2000[40]), which may be important to orchestrate coping behaviors towards predators in an anticipatory response that might avoid a direct encounter.

A parallel circuit, comprising the same brain structures, orchestrates

defense responses towards conspecifics. Pheromonal and olfactory information from conspecifics activate the posterior dorsal MeA (pdMeA) that projects to the ventrolateral VMH (vlVMH) (Gross C 2012[2]). In mice, optogenetic activation of the vlVMH triggers aggressive behaviors towards both male and female intruders, as well as towards inanimate objects (Lin D 2011[56]). The vlVMH and the dorsal medial premammilary nucleus (dmPMD) are part of the conspecific-responsive circuit in the hypothalamus. The introduction of an intruder mouse in the homecage of another mouse, leads to the display of several defense reactions that can be either passive (freezing, on-the back position) or active (upright standing, boxing…). It has been found that this interaction increases *c-fos* expression in the dmPMD of the intruder but not of the resident. Also, intruders with lesions in this subnucleus showed a major deficit in passive defense behaviors, while keeping certain key active responses (Motta SC 2009[57]). The dPAG also seems to be important for the display of coping strategies towards conspecifics, given that pharmacological inhibition of this area in mice reduced defense responses when facing an aggressive mouse (Silva BA 2013[55]). The segregation between predator and conspecific defense neuronal circuits may also be kept in the PAG, since the dmPMD projects mainly to the dorsomedial PAG (dmPAG) (Gross C 2012[2]).

Concerning innate responses triggered by sounds, it has recently been reported that innate flight can be induced by a broadband white noise sound of high intensity. Interestingly, the authors report that a similar response can also be induced by a loud 5Khz pure tone, suggesting that flight responses can be generally induced by loud sounds. The authors found that the shell region of the Inferior Culliculus (ICC) relay ascending auditory inputs to the Auditory Cortex through the Medial Geniculate Body (MGB). Optogenetic activation of

the axonal terminals of the corticofugal neurons of the Auditory Cortex, targeting the cortical ICC, is sufficient to induce flight. The escape response is mediated by the inputs from the cortical ICC to the dPAG (Xiong XR 2015[52]).

It has also been shown that a looming stimulus, an expanding dark disc, which simulates an approaching threat from above the animal, can induce visually innate fear responses such as escape and freezing (Yilmaz M 2013[49]). Recent work focusing on the display of freezing triggered by this stimulus in mice, revealed that a subcortical pathway from the medial inferior layer of the Superior Culliculus (SC) to the lateral posterior nucleus of the Thalamus and forward to the LA, mediates visually evoked innate freezing (Wei P 2015[51]). Interestingly, another study reported that optogenetic activation of parvalbumin positive neurons in the SC (SC PV^+) triggers impulsive escape followed by long lasting freezing in mice. The authors report that light induced activation of SC PV^+ axonal terminals in the parabigeminal nucleus is sufficient to trigger the defense behaviors. Given the projections from the parabigeminal nucleus to the amygdala, in particular to the central nucleus, the authors propose that SC PV^+ neurons form a subcortical visual pathway that transmits threat relevant information to the Amygdala (Shang C 2015[50]). Although these two works report different pathways underlying visually evoked innate defense responses, they provide evidence that the Amygdala is necessary for the display of innate defense behaviors triggered by visual stimuli.

As previously referred, painful/noxious somatosensory stimuli also leads to the display of defense behaviors. In rodents, the delivery of an electric shock elicits an initial burst of motor activity, which may include running, jumping, ultrasonic vocalizations (USVs) or fast head movements (Fanselow MS 1998[58],

Blair H 2005[59]). These initial motor responses are in general followed by stretch positions and immobility in enclosed spaces, defense behaviors that are conditioned to the environment. Both the spinothalamic and the spino-parabrachial tracts transmit nociceptive information from the periphery to the forebrain including the Amygdala. This information can be sent both directly through the spinothalamic and the spino-parabrachial tract (Kruger L 1998[60], Han S 2015[61], Cliffer KD 1991[62]) and indirectly through the paraventricular nucleus of the Thalamus (Penzo MA 2015[63]) to the lateral portion of the CeA (CeL). The LA also receives nociceptive information indirectly through the somatosensory thalamus and cortex (Lanuza E 2004[64], Bubser M 1999[65]). Bilateral electrolytic lesions of the posterior intralaminar thalamic complex destroy fibers from both these tracts, leading to the disruption of freezing after footshock delivery (Lanuza E 2004[64]). Moreover, pretraining lesions of both the posterior intralaminar thalamic nucleus (PIN) and the insular cortex significantly attenuated the magnitude of shock-induced activity in lesioned rats compared to controls (Shi C 1999[66]).

A recent study shows that a population of calcitonin gene-related peptide (CGRP) expressing neurons in the lateral subdivision of the parabrachial nucleus is anatomically and functionally connected to CGRP neurons in the CeL. The authors show that functional silencing of CGRP neurons in both the parabrachial nucleus and CeL during footshock delivery blocked the defense escape behaviors triggered by shocks, suggesting that activity in these neurons is important for the pain signaling. This work suggests that the parabrachial nucleus transmits information about the aversive footshock to neurons in CeL, and further experiments confirmed that this pathway is necessary for fear learning induced by footsocks (Han S 2015[61]).

Nociceptive information about footshocks can also be conveyed to different subnuclei of the amygdala through the paraventricular nucleus of the Thalamus. Footshock stimulation leads to a significant increase in *c-fos* expressing neurons in this region (Penzo MA 2015[63]). In addition, a study combining immunohistochemistry with retrograde tracing revealed that *c-fos* expressing cells in this thalamic nucleus, in response to footshocks, project to the CeA and BL subnuclei, as well as to the Prefrontal Cortex and Nucleus Accumbens (Bubser M 1999[65]).

The LA is also proposed to be an important site for processing aversive nociceptive information. Footshock stimulation in an enclosed box leads to an increase of *c-fos* expression in all subnuclei of the LA, when compared to animals just exposed to the box. Unilateral electrolytic lesions of the PIN and the medial division of the MGB (MGm) significantly decreased the number of *c-fos* labeled cells in LA after footshock when compared with intact animals. This result suggests that the LA is involved in the processing of footshocks and that the somatosensory information is provided by the MGm and PIN (Lanuza E 2008[67]). It has also been shown that electrolytic lesions and muscimol inactivation of the LA reduced the unconditioned response of head movement to the presentation of an eyelid shock (Blair H 2005[59]). In a different study using a similar paradigm, it was also shown that cells in the LA respond to eyelid shock delivery. The authors also report that muscimol inactivation of the PAG greatly reduced these responses, and it was found that cells in different columns of the PAG also respond to this aversive nociceptive stimulus. These results suggest that the PAG may participate in relaying information about the aversive stimuli to the LA (Johansen J 2010[68]).

A recent study using a transgene where the expression of the fused protein Channelrhodopsin2 (ChR2)/Yellow Fluorescent Protein (YFP) is under

the control of a *c-fos* promoter showed that footshock stimulation triggers neuronal activity in the BL resulting in the expression of the fused protein in 3% of the neurons. ChR2 is a light gated channel, whose activation by light leads to neuronal depolarization (Nagel G 2003[69]). Optical excitation of the footshock responsive neurons expressing ChR2/YFP decreased both heart and respiration rate, and increased the levels of freezing compared with controls where ChR2/YFP was expressed in a random population of neurons (Gore F 2015[70]).

Together, these results suggest that information about nociceptive aversive stimulus like a footshock is transmitted to both BL and CeA.

The examples above illustrate that the same defense behaviors (namely freezing, flight or fight) can be triggered by innately aversive stimuli of different sensory modalities, and through different neuronal pathways. The defense responses triggered by such cues are activated from the first encounter with the threat, supporting the idea of developmentally programmed neuronal circuits devoted to rapidly responding to specific threats in the environment. Importantly, such cues can underlie fear learning about other cues that are not innately aversive. In the next section we will focus on the neuronal mechanisms of fear learning, with emphasis on studies that used footshocks as an innately aversive stimulus. This stimulus has been widely used under laboratory settings given that it is easily controlled by the experimenter and induces robust fear learning.

b) Learned Defense Responses

As previously mentioned, coping strategies can be evoked by a stimulus that is not intrinsically aversive, as long as this stimulus was previously paired with an innate threat. One paradigm that has been extensively used in laboratory studies to assess the mechanisms of learned fear responses is Auditory Fear Conditioning (AFC). In this paradigm, an initial neutral stimulus, like a pure tone, is paired with an innately aversive stimulus like a footshock (termed unconditioned stimulus US). An association is made between the two, and the animal learns that the presentation of the sound (now the Conditioned Stimulus CS) predicts the US. The presentation of the CS alone is now sufficient to trigger defense behaviors.

Behavioral, anatomical and physiological studies based on this paradigm revealed cellular mechanisms as well the neuronal circuits underlying aversive learning towards an auditory cue. There is extensive literature showing that the Amygdala is a key structure for fear learning and memory (reviewed in Maren S 2004[43], Pape H 2010[42], Herry C 2014[7]). In what concerns the neuronal pathways providing auditory information to the Amygdala, it has been shown that several subnuclei of the auditory thalamus project directly to the LA (Neot DD 1999[71]), with the exception of the ventral MGB (MGv) (the primary input to Auditory Cortex area 1). These nuclei also project to the Auditory Cortex (Smith PH 2012[72], Kimura A 2003[73]), and cortical projections to LA originate in the secondary auditory and perirhinal cortex (Romanski LM 1993[74], McDonald AJ 1998[75]). Hence, information about an auditory stimulus arrives to LA through both direct thalamic and indirect cortical pathways, and inputs from both areas contribute to auditory fear conditioning. However, it has also been shown that these two pathways are not redundant (Romanski LM 1992[76], Campeau S 1995[77], Jarrel TW 1987[78], Johnson LR 2011[79], Antunes R

2010[80]). Importantly, is has been reported that after AFC, CS-evoked responses are enhanced in cells both in the MGm of the auditory thalamus and in auditory cortical areas, showing that plasticity occurs in the pathways that provide CS information to the LA (for review see Maren S 2004[43], Herry C 2014[7], Ehrlich I 2009[81]).

Several studies have demonstrated that the LA is necessary for learning, storage and expression of defense behaviors triggered by sounds (Hitchcock J 1986[82], Hitchcock J 1987[83], LeDoux JE 1990[84], Romanski LM 1993[74], Quirk GJ 1995[85], Schafe GE 2005[86], Rumpel S 2005[87], Han JH 2009[88], Johansen J. 2010[68], Gouty-Colomer LA 2015[89]). Some of the first evidence that LA is an important site for the acquisition of auditory fear learning was provided by studies performing electrolytic lesions in this nucleus (Hitchcock J 1986[82], Hitchcock J 1987[83], LeDoux JE 1990[84]). These studies showed that animals with LA lesions have impaired auditory fear learning, since both potentiated startle and freezing responses to the presentation of the conditioned CS are decreased when compared with intact animals. Its role in integrating information of different sensory modalities has been elucidated by electrophysiology studies showing that cells in LA receive convergent inputs from both the CS and the US (Romanski LM 1993[74]). Both pharmacological manipulations and electrophysiology recordings provided evidence of synaptic plasticity in this nucleus. It has been reported that AFC increases CS-evoked responses in LA (Quirk GJ 1995[85], Rogan MT 1997[90], Repa JC 2001[91], Johansen J 2010[68]), which is consistent with conditioning induced changes in auditory responses. Pharmacological blockade of N-methyl-D-aspartate (NMDA) receptors (Rodrigues SM 2001[92], Miserendino MJ 1990[93]) or intracellular signaling cascades in LA impair fear memory acquisition and

consolidation (Schafe GE 2005[86], Schafe GE 2000[94]). Together, these results show that synaptic plasticity in this nucleus underlies auditory fear memory.

The role of LA in fear memory storage has also been shown by both lesion and molecular studies (Maren S 1996[95], Han JH 2009[88]). Taking advantage of previous findings (Han JH 2007[96]) showing that LA neurons overexpressing cyclic adenosine monophosphate response element–binding protein (CREB) were preferentially activated during fear conditioning compared with neurons with non-altered CREB expression, the authors used an inducible diphtheria-toxin strategy to specifically ablate CREB overexpressing neurons after fear learning. This manipulation significantly blocked expression of the fear memory and this loss was persistent over time, suggesting that the ablation of a specific neuronal subpopulation in LA is sufficient to permanently abolish an aversive memory (Han JE 2009[88]).

The US-evoked depolarization of pyramidal cells in LA is thought to underlie hebbian plasticity, by favoring synaptic association between neurons that respond to the US and afferents with a concomitant, although weaker, CS-evoked response. This hypothesis has been tested by expressing ChR2 in LA pyramidal cells of rats that were subjected to an AFC task where the CS coterminated with light activation of these neurons instead of a footshock. This pairing was sufficient to support fear learning (Johansen J 2010[68]).

Changes in firing rate due to fear learning have also been shown in other nuclei in amygdala. The LA sends strong projections to the BL and a study performed in mice showed that cells in this nucleus showed increased initial phasic responses to the presentation of the CS after FC (Herry C 2008[97]). A posterior study in rats unraveled that subsets of neurons in the BM and BL subnucleus acquire increased sustained responses throughout, and in the BM

even outlasting, the presentation of the CS. These results suggest that neurons in the BM nucleus are not passive relays of the phasic responses seen in LA. Importantly, inactivation of both BM and BL decreased fear expression in a testing session, revealing the importance of basal nucleus for the display of acquired defense behaviors in response to an initial neutral cue (Amano T 2011[98]). Importantly, a recent study reported that footshock stimulation triggers neuronal activity in the BL in mice. Optogenetic stimulation of ChR2/YFP protein expressed in neurons activated by the footshock was sufficient to trigger defensive behaviors as well as drive auditory fear learning (Gore F 2015[70]).

The expression of conditioned defense behaviors to noxious stimuli such as footshocks is believed to be under the control of CeA. Interestingly, lesions of this nucleus disrupt aversive learning supported by footshocks, but don't interfere with conditioned defense responses to a predator (Gross C 2012[2], Martinez M 2011[54]).

The CeA is classically viewed as a relay between the basolateral complex and the hypothalamic, midbrain, and brainstem systems. Electrolytic lesions of its downstream targets, the lateral hypothalamic nucleus and the PAG, reduce respectively the increase in mean arterial pressure and freezing to the CS (LeDoux JE 1998[99]). The basolateral complex of the Amygdala project directly or indirectly through GABAergic intercalated neurons to CeA. This nucleus has been further subdivided into the medial (CeM) and lateral (CeL) subnuclei. Optogenetic activation of CeM drives freezing, as well as inhibition of the CeL, indicating that neuronal activity in CeM is sufficient to drive defense behaviors, and is under inhibitory control of CeL. Muscimol inactivation of the CeL but not CeM during AFC results in impaired memory retrieval 24h later, suggesting that

activity dependent neuronal plasticity in CeL is necessary for fear memory acquisition (Ciocchi S 2010[100]). This and other studies (Duvarci S 2011[101], Han S 2015[61], Herry C 2014[7]) contributed to the idea that CeL is mostly involved in fear acquisition, while activity in CeM is closely related with fear expression (but see Penzo MA 2013[102], Penzo MA 2015[63]). Given that most of the brainstem projecting cells is concentrated in CeM, this subnulceus is thought to be the main output to downstream effector targets.

In support of this view, distinct neuronal populations in CeM differentially affect the physiological and behavioral components of a defensive response. Namely, neuronal activity in cells that project to the dorsal vagal complex modulate changes in heart rate when an animal is exposed to a previously learned threatening environment. On the other hand, intermingled cells in CeM that project to the vPAG affect the expression of freezing. Inhibition of these later cells by oxytocin significantly decreases freezing but has no effect in the cardiovascular component (Viviani D 2011[103]).

Interestingly, the classical view that the PAG is just an output station downstream of Amygdala has been recently challenged. Pairing dPAG stimulation with an auditory CS is sufficient to support AFC; however, if BL is inhibited, conditioning does not occur. This data suggests that BL may be downstream target of dPAG in aversive auditory learning (Kim E 2013[104]). Also, pharmacological inactivation of PAG reduces shock-evoked responses in LA and the acquisition of aversive learning (Johansen J 2010[68]). This data suggest a new role for the PAG as a potential source of information about the aversive stimuli, providing the amygdala with instructive information about the threat.

Together, these studies identify the Amygdala as an important locus for fear learning and storage when the US is an aversive nociceptive stimulus. They also contributed to a better understanding of the mechanisms underlying synaptic plasticity, and created a framework of how to assess other learned defensive behaviors.

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Part II - Social Transmission of Fear

" *Neither the mouse nor the gazelle can afford to learn to avoid; survival is too urgent, the opportunity to learn is too limited, and the parameters of the situation make the necessary learning impossible"*

Robert C. Bolles "Species-specific defence reactions and avoidance learning"

The above statement by Bolles R.C. points out that in natural populations the costs of learning by self-experience about the threats in the environment can be too high, risking the survival of the individual. The existence of hardwire neuronal pathways that allow the innate recognition of predators is then extremely advantageous since they underlie the display of defense responses towards the threat. Importantly, even if a given individual is able to recognize a threat, it may not detect it until the threat is in close proximity. Social information that signals impending danger has been shown to both increase alertness (Wilson DR 2004[1], Ito R 2011[2]) and trigger defense behaviors in observer animals (Seyfarth RM 1980[3]). This phenomenon is described as social transmission of fear and most likely is of major importance in threat avoidance. Given that it does not require the direct detection of the threat but the use of information conveyed by others, it is likely to decrease the risk of direct encounters with a predator. However, it implies that at least one of the individuals in the environment is able to recognize the threat, either innately or subsequently to learning.

Importantly, predation risk can vary in space and time, and environmental changes can alter the landscape of predators to which the animals are exposed. The introduction of novel predators can carry high risks for the

survival of indigenous preys since these species may fail to recognize cues or hunting strategies from novel invasive species (Gomez-Mestre I 2001[4], Polo Cavia N 2010[5], Berger J 2011[6]). The ability to learn to associate novel cues to threatening situations is then advantageous in dynamic environments. Currently, there are several pieces of evidence that learning plays an important role in developing defense responses towards threats. For the particular case of predator avoidance this learning may rely on social information (reviewed in Griffin AS 2004[7]).

Defense responses towards predators can therefore be acquired through social learning. According to Heyes', "Social learning refers to learning about other agents or the inanimate world that is influenced by observation of, or interaction with, another individual or its products. These products can include deposits, such as scent marks, and the effects of actions on objects and environments" (Heyes C 2012[8]). For the particular case of social learning about threats, many authors pointed out that the mechanisms underlying it are similar to those of Pavlovian Stimulus-Stimulus association (Mineka S 1993[9], Griffin AS 2004[7], Heyes C 2012[8]). In the case of observational fear conditioning, the fear displayed by a demonstrator animal in response to a fear eliciting stimuli (whether innate or previously learned) can serve as unconditional stimuli (US) and hence trigger unconditioned responses (UR) in the observer. The later makes a new association between the stimulus that elicited fear in the demonstrator and its' own UR. In consequence, the stimulus that triggers defense responses in the demonstrator is now also a conditioned stimulus (CS) to the observer, leading to the expression of defense conditioned responses (CR). In a study addressing this hypothesis, Mineka *et al* (Mineka S 1993[9]) exposed an observer monkey to the fear responses triggered by a

snake in a model monkey. Importantly, observer monkeys didn't respond fearfully to the snake prior to the experiment. The authors found a strong relationship between the models' fear of snakes and the acquired fear of the observers tested posteriorly. Interestingly, during the conditioning session there was a very high correlation between the disturbance triggered by the snake in the model and the disturbance in the observer monkey. This is in agreement with the idea that the fear responses of a demonstrator act as an US that triggers UR in the observer.

An essential part of observational fear is then the perception of social information that can be accessed through different sensory modalities. Whether there are mechanisms and neuronal circuits devoted to social information is still a matter of debate (Adolphs R 2010[10]).

Summing up, during social transmission of fear there is an initial direct recognition of a threat by one or more individuals in a group, that underlies behavioral and physiological changes in these animals (demonstrators). Such changes can be perceived by others (either con or heterospecifics) causing neuronal and behavioral alterations that underlie avoidance responses in observers. Importantly, for social transmission of fear to occur it is not necessary that the observer recognize the threat that triggered the defense behavior in others. If such recognition occurs, social transmission of fear may underlie social learning about threats through an association between the defense behaviors of demonstrators and the threat that triggered them. This learning process will allow that witnesses in future encounters with a previously unknown threat directly recognize the impending danger.

In the context of the following thesis we focused on the mechanisms of social transmission of fear. On one hand, studying this behavior addresses an important ecological phenomenon that may deeply influence survival. A better understanding of how information is transferred between individuals in the context of fear will also shed light into the mechanisms of social learning about threats. Studying this phenomenon in laboratory settings will also contribute to unravel the neuronal mechanism underlying it and increase the knowledge about how social information is processed in the brain. Finally, it creates a framework to study how individuals integrate information acquired by self-experience with information conveyed by others, which is of major importance to understand group dynamics.

In the next sections we will review the literature on social transmission of fear in several species and in both natural or laboratory settings. To avoid confounds given the different nomenclature in these studies, we will designate by demonstrator the animal that display the fear responses triggered by a direct recognition of either an innate or learned aversive cue. The witnesses of such display will be designated observers.

II.I Sensory Cues underlying Social Transmission of Fear

Transmission of fear between conspecifics has been reported in many species, ranging from invertebrates to birds, fish and mammals (Ono M[11], Hingee M 2009[12], Cornell HN 2011[13], Coleman SW 2008 [14], Mirza RS 2003[15], Zuberbuhler K 2001[16], Wilson DR 2004[1], Hollen LI 2009[17], Enjin A 2013[18]).

The recognition of behavioral and physiological changes in others can be achieved through different sensory modalities including olfaction, audition and vision.

Chemical communication is quite pervasive and a wide range of products like pheromones (intraspecies cues), kairomones (interspecies cues that benefit the recipient) and allomones (interspecies cues that benefit the emitter) have been mentioned in animal communication. The secretion of pheromones can convey information about the releaser, namely sex, social status and reproductive state. These chemicals can be detected through the olfactory and gustatory system and can influence the physiology and behavior of the individual that encounters them in a variety of ways. As an example, many species secrete chemicals in threatening situations termed alarm pheromones that trigger defense behaviors like freezing, attack or disperse in conspecifics (Ferrero DM 2010[19], Beni Y 2014[20]).

Chen and Li found that the weaver ant *O. smaragdina* preys giant honeybees *A. dorsata* while the latter are foraging for nectar under flowers. When the honeybees perceive the threat, they fly away from the risky plants leaving an alarm pheromone that prevents other bees to visit the flowers were the attacks took place (Chen & Li 2012[21]).

Defense responses triggered by chemical cues have also been shown in fish. The exposure of juvenile yellow perch, *Perca flavescens*, to damage-released alarm cues from injured conspecifics triggered antipredator responses like an increased use of a shelter and freezing (Mirza RS 2003[15]).

The use of auditory information conveyed by others is also quite prevalent, and acoustic signals can be highly advantageous given that they spread very rapidly. Also, they can be easily detected by members of the group that are nearby, but not necessarily in the sight of the individual that detects the threat. Common sources of acoustic signals are alarm calls emitted when an individual

detects a threat (Hollen LI 2009[17]). Vervet monkeys (*Cercopithecus aethiops*) emit alarm calls that are specific to the approaching predator, triggering defense behaviors in witnesses that are the most adaptive given the threat. Namely, if monkeys are on the ground, calls to leopards trigger an escape to trees. If the call was triggered by the detection of an eagle, witnesses mainly run to a cover. Playback experiments confirmed these results, and showed that acoustic cues are sufficient to trigger defense behaviors (Seyfarth RM 1980[3]).

Adult Belding's ground squirrels (*Spermophilus beldingi*) also produce two distinct types of audible vocalizations given the detected threat, and this calls elicits different responses in other individuals of the group. A study reports that responses to these alarm calls develop during juveniles early life, since by the time of emergence from the burrows the young don't distinguish between different alarm vocalization (Mateo JM 1995[22]). On another rodent species, it was also shown that Richardson's ground squirrels (*Spermophilus richardsonii*) whisper calls that contain pure ultrasonic frequencies around 50Khz. When exposed to playbacks of these calls, animals spent significantly more time in vigilant behavior than in response to a background control sound. The authors suggest that these calls may be used to selectively warn individuals of their kin while remaining undetected by predators (Wilson DR 2004[1]).

Interestingly, it has also been shown that acoustic signals other than alarm calls can trigger defense behaviors in observers (Hingee M 2009[12], Coleman SW 2008[14], Randall 2001[23]). In a recent study (Hingee M 2009[12]), it was found that the presentation of a threat to crested pigeons triggers an alarm take off flight whose whistles are louder and with a faster tempo than the ones resulting from a normal take off. The authors propose that the acoustic differences result from the vibration of a highly modified eight primary feather, suggesting that more than a by-product of flight this acoustic cue can be a

signal of danger. When playing back the sound of alarm and normal take offs to conspecifics, it was found that they used these acoustic differences adaptively since they take off in alarm only after alarmed whistles.

As previously referred, the direct observation of the defense responses of a conspecific can trigger fear in observer monkeys (Mineka S 1993[9]). The importance of visual information has also been documented in humans. In a laboratory experiment, participants undergoing a differential fear-conditioning task were filmed. These participants (learning model) were presented with two squares of different colors that served as CS, being the presentation of one of the CS (CS+) coterminated with a mild aversive shock delivered to the wrist. A different group of subjects was afterwards presented to the video showing the learning model during the conditioning session. Skin Conductance Responses (SCR) (used as indicators of physiological and psychological arousal) where measured in subjects while they watched the movie, and there was a significant increase in SCR when the US was delivered to the learning model. In a posterior test phase, the authors also found a significant increase in SCR when subjects were presented with the CS+ without shock delivery. These results indicate an increased autonomic response when witnessing the distress of others, and that subjects learned about the CS/US contingency. Given that the stimulus used was a video, it also supports the idea that facial expressions of distress can serve as an aversive US (Olsson A 2007[24]).

The above examples show how diverse can be the information used by individuals in a group to detect cues that signal danger provided by those surrounding them. Understanding how this information is processed in the brain, both at the sensory periphery and in downstream circuits recognized to

be involved in defense behaviors, is essential to understand how observational fear is triggered. However, such analysis is virtually impossible in natural populations. Animal models classically used in laboratory settings can be quite useful given the amount of techniques currently available and optimized for such models, and the possibility to perform experiments in controlled environments. Mice and rats are commonly used in laboratory settings, and there is a growing body of evidence of social communication in species kept in laboratories.

II.II Sensory Stimuli in Intraspecies Communication in Rodents

As previously mentioned, chemical cues play a very important role in animal communication. In rodents, odors are detected by olfactory sensory neurons located in different olfactory tissues, namely the Main Olfactory Epithelium, Vomeronasal Organ, the Grunenberg Ganglion and the Septal Organ of Massera (Ferrero DM 2010[19]).

The Vomeronasal Organ, the Grunenberg Ganglion and the Main Olfactory Epithelium have been implicated in the detection of pheromones, which can be either small volatile molecules or non-volatile peptides. These compounds can be released from a wide range of body secretions (like urine and preputial gland secretion) (Beny Y 2014[20]).

The role of alarm pheromones in triggering defense behaviors has been described. Subjecting mice to extremely stressful situations leads to the release of a water soluble alarm pheromone. The delivery of this chemical stimulus to brain slices leads to a significant increase in calcium in the Grunenberg Ganglion cells. In vivo experiments showed that exposing intact mice to such alarm pheromone in a closed box induces freezing and a decrease in walking distance. Lesion of the Grunenberg Ganglion abolished the

defense behaviors, showing that this olfactory subsystem mediates alarm pheromone detection (Brechbuhl J 2008[25]).

In rats, the delivery of footshocks leads to the release of alarm pheromones that trigger both autonomic and behavioral changes in conspecifics. Electrical stimulation of the whisker pads releases a chemical cue that enhances risk-assessment, while the one released from the perianal gland triggers both stress induced hyperthermia (Kiyokawa Y 2004[26]) and defense behaviors (Kiyokawa Y 2006[27]) in receiver rats. The exposure to this latter pheromone leads to increased *c-fos* expression in several brain nuclei involved in stress and fear, namely the bed nucleus of the stria terminalis, paraventricular nucleus, medial (MeA) and basolateral subnuclei (BLA) of amygdala and ventral division of the Periacqueductal Gray (vPAG) (Kiyokawa Y 2005[28]). Importantly, this pheromone is water soluble and volatile, affecting behavioral responses of rats exposed to it at small but not long distances (Inagaki H 2009 [29]). It has also been recently found that it is detected by the Vomeronasal Organ, and the ablation of this organ abolished behavioral responses to this compound in different assays (Kiyokawa Y 2013[30]).

The role of auditory information, namely vocalizations emitted by conspecifics, as also been reported in laboratory settings. Rats emit ultrasonic vocalizations (USVs) categorized in 3 groups: emitted by pups in social isolation, emitted by adults and juveniles in aversive situations such as predator exposure and fighting (distress 22Khz USVs), and emitted by juveniles and adults in appetitive situations like play and mating (afiliative 50 kHz USV).

Given the situations in which they are emitted, it is believed that 22 kHz USVs reflect a negative affective state related with fear and anxiety. The presentation of an aversive US during fear conditioning triggers the emission of 22kHz USVs. The emission of these calls is also a prominent part of rats CRs to the

presentation of the CS, and lesions of the central amygdala (CeA) significantly reduce their emission both during aversive learning and recall (Wohr M 2013[31], Choi J 2003[32]).

The most widely accepted biological function for 22 Khz USVs is that they serve as alarm calls to warn conspecifics about external danger. This hypothesis is mainly supported by studies conducted by Blanchard *et al.* (Blanchard RJ 1991[33]) where rats were presented to a cat either in a visible burrow system together with their colony, or alone in an open field. It was found that the percentage of time rats spend vocalizing is significantly higher in the visible burrow system, suggesting that the presence of an audience facilitates the emission of these calls. However, a more recent study failed to see evidence of an audience effect. Rats were conditioned and tested to a tone either in the presence or absence of a cagemate. Alarm calls were emitted at both experimental periods, but were not potentiated by the presence of a familiar conspecific (Wohr M 2008[34]). These apparently opposing results cannot solve the question of whether or not alarm calls are emitted to warn conspecifics, since the discrepancy can be due to differences in the experimental apparatus and housing condition. Further studies were done to clarify this question, looking at defense behaviors triggered by the playback of natural 22kHz USVs. Most studies found only weak or no behavioral responses at all, indicating that alarm calls are probably not aversive US (Sadananda M 2008[35], Parsana AJ 2012[36], Worh M 2013[31]). However, studies where 22kHz were used as CS suggest that the behavioral responses found to this acoustic stimuli emerge from associative learning, and that there is a facilitated predisposition for such association (Bang SJ 2008[37], Wohr M 2013[31]).

In an attempt to better understand the possible negative valence of these calls, studies looked at the neuronal responses in brain areas that

regulate fear and stress. Exposing animals to 22kHz alarm calls lead to a significant increase in the number of c-fos labeled cells in lateral amygdala (LA), BL, Perirhinal Cortex and the dorsomedial part of the PAG (dmPAG), when compared with animals exposed to affiliative calls or not exposed to any sound (Sadananda M 2008[35]). A more detailed characterization of amygdala responses to USVs showed that cells in LA respond to both 22kHz and 50kHz vocalizations, as well as to pure tones of same frequency. Even tough the percentage of cells responding to these stimuli is similar, 22kHz stimuli triggered mostly tonic excitatory responses while 50kHz triggered mostly tonic inhibitory responses. The authors suggest that this bidirectional tonic activation to negative and positive social signals can be a sensitive index of emotional valence (Parsana AJ 2011[38]).

Although research in social communication in rodents focus mainly in olfactory or auditory cues, there is also evidence that visual cues may play an important role.

In a study addressing whether the online observation of pain in a conspecific can influence ones own display of pain, Langford *et al.* (Langford DJ 2006[39]) injected a noxious stimuli (acetic acid 0.9%) that causes abdominal constriction (writhing) in a mouse that was either isolated or in a dyad with a conspecific. The conspecific received or not the same aversive stimuli. They found that mice that were paired with cagemates that also received the noxious treatment displayed significantly more writhing than isolated mice. When probing for sensory cues, the only manipulation that significantly decreased hyperalgesia caused by the noxious treatment of both mice was an opaque plexiglass barrier. This result suggests that visual information about a conspecific's pain can affect an observer's display of pain.

In humans, facial expressions of almost every emotion have been characterized, and are important in social communication. However, such characterization in other species other than humans is quite sparse. By using the same kind of pain manipulation mentioned in the previous study, researchers recorded and characterized facial expressions of individual mouse in pain (Langford DJ 2010[40]). From this characterization resulted a mouse grimace scale consisting of five facial features: orbital tightening, nose bulge, cheek bulge, ear position and whisker change. A recent study by Nakashima *et al.* accessed if these expressions have a communicative function (Nakashima 2015[41]). In this study, the researchers used an apparatus with 3 compartments, a central zone and two lateral chambers. The walls of one of the chambers had pictures depicting rats with facial expressions of pain while in the other the expressions were neutral. Rats tested in such environment spent significantly more time in the compartment with neutral expressions, suggesting that rats are able to discriminate visual stimuli that corresponds to facial expression of different emotional states.

II.III Social Transmission of Fear in the Lab

The ability of rodents to detect and react to defense behaviors of others led to a recent increase in studies performed under laboratory settings. Behavioral tasks have been carefully designed in order to address in which conditions social transmission of distress cues occurs, how sensory information provided by the fearful conspecific is perceived and which neuronal circuits are responsible for a change in the behavior of the observer subject.

The interaction between a fearful conspecific and the observer can influence the behavior of the latter in different ways. The exposure of an observer to a fearful conspecific may lead to a transfer of emotional information between

subjects. If such interaction occurs before the observer engages in a fear-learning task it may influence the subsequent learning through social modulation. On the other hand, social transmission of fear occurs if an observer directly witnesses the defense behaviors of the demonstrator and this observation triggers defense behaviors in the observer. If posteriorly to this interaction the exposure of the observer alone to the cue that triggered fear in the demonstrator also leads to the display of defense behaviors in the observer, we are in the presence of social learning about threats.

The effects of an interaction between a naïve rat and a previously fear conditioned conspecific (demonstrator) were reported by Knapska *et al.* (Knapska E 2006[42]). The authors found that reuniting the demonstrator with a cagemate (observer) in their homecage increased the exploration of the demonstrator by the observer. This change in behavior was accompanied by an increase in the number of *c-fos* positive cells in several amygdala sub-nuclei namely LA, BL, BM and MeA in both animals when compared to controls (Knapska E 2006[42]). A follow up of this study showed that such interaction prior to a shock-motivated shuttle avoidance task facilitated learning by observer rats (Knapska E 2010[43]). Interestingly, another study performed in mice looking at social modulation of fear learning found that the prior exposure of a mouse to a recently fear conditioned conspecific impairs the acquisition of conditioned fear to an auditory cue. Similar results were achieved if the observer was exposed to an olfactory chemosignal from a recently fear-conditioned familiar mouse or a putative anxiogenic pheromone, b-phenylethyl-amine (b-PEA) (Bredy TW 2009[44]).

While in the previous experiments the interaction between the observer and the fearful demonstrator only happened after the aversive event, other studies looked at the behavioral changes induced by directly witnessing the distress of others. Chen Q. *et al.* (Chen Q 2009[45]) exposed observer mice of two different strains (B6 and BALB-C) to another mouse being fear conditioned to a tone (CS). Afterwards, observer mice were tested to the CS and tone-shock conditioned (CS-US pairing). The authors found that the pre-exposure to the distress of others affected differently the two strains, with B6 mice showing a significant increase in freezing to the CS when compared with BALB-C. There was also a significant difference between these two strains in the acquisition of conditioned fear during the exposure to the CS-US, with an enhanced acquisition for the BALB-C. These results suggest that the genetic background may influence both social learning and social modulation of fear acquisition. One interesting aspect of this work is that while demonstrator mice were fear conditioned, the objects oriented but didn't freeze in response to the distress of others.

In a study looking at social modulation of avoidance, the authors report that rats that received a footshock in a dark chamber and then witness another rat being shocked (demonstrator) in the same environment, have an increase latency to enter this chamber than rats tested alone. Importantly, after the demonstrator is shocked it returns next to the observer rat, suggesting that not only the direct witnessing of the shock but also the posterior interaction with the fearful conspecific can contribute to the increased latency. Interestingly, if the observer never experienced footshocks in such chamber, the presence of a fearful conspecific doesn't significantly increase its latency to enter the chamber. These results suggest that not only Pavlovian fear conditioning but also avoidance can be socially modulated. Importantly, this

modulation occurred only with avoidance-experienced rats, suggesting that a prior similar aversive experience may by necessary for this modulation to occur (Masuda A 2009[46]).

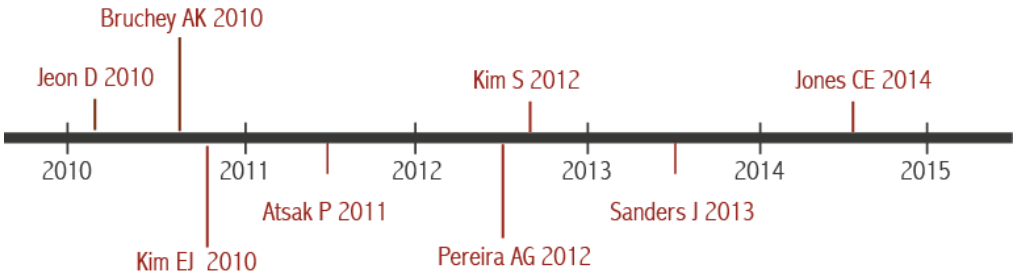
A prior study looking at suppression of lever pressing by witnessing pain of conspecifics found similar results. Church MR (Church MR 1959[47]) showed that rats' suppressed lever pressing for food while witnessing a demonstrator being footshocked, given that they had a prior aversive experience with shocks.

The role of prior aversive experience has also been shown in social transmission of fear in a Pavlovian fear-conditioning task. In a set of experiments where a rat witnesses a conspecific being footshocked (Atsak P 2011[48]), the authors found that the observers expressed observational freezing only if they had prior experience with footshocks.

These previous studies show that exposure to a fearful conspecific can either affect fear learning and/or trigger defense behaviors in observers. However, very little is known about the neuronal mechanism underlying social modulation, learning or transmission of fear. With the intention to focus on transmission of fear, we adapted the behavioral paradigm developed by Atsak P *et al.* (Atsak P 2011[48]) by looking at transmission of fear during the recall of the aversive memory by the demonstrator and not during fear acquisition. The intention was to isolate the fear responses from the pain induced by the footshocks. With this new paradigm we planned to test whether prior experience was also necessary even in the absence of pain. Furthermore, we looked at the sensory cues underlying the behavior with the purpose to better understand the nature of social information used to detect defense behaviors in conspecifics. Also, the identification of the sensory cues necessary for social transmission of fear provides an indication of the neuronal circuits involved in

this behavior. The last goal was to investigate the neuronal structures involved in the display of defense behaviors triggered by fearful conspecifics and to investigate the brain regions involved in the detection of the sensory cues provided by them.

Importantly, during the time course of this work other studies addressed questions similar to ours. These studies will be referred and discussed in further detail in the final discussion.



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CHAPTER 2 - Fearful Silence: rats use the Cessation of Movement-evoked Sound to Detect Danger.

"As a rule, prey animals try to hide their presence by adaptative silence"

Eberhard Curio in *"The ethology of predation" 1976*

Acknowledgements

Marta Moita and Ana Pereira designed the experiments in this chapter with the help of Susana Lima and Andreia Cruz. Ana Pereira performed all the experiments and analyzed the data. Ana Pereira, Marta Moita, Susana Lima and Andreia Cruz discussed the results.

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ABSTRACT

It is well documented in natural populations that social information can be used to signal danger. Most of these studies have focused on the use of private channels of communication such as alarm pheromones and alarm calls (Mirza RS 2003[1], Ono M 2003[2], Wilson DR 2004[3], Platzen D 2005[4], Zuberbuhler K 2001[5]). However, there are very few reports of transmission of fear between con-specifics under laboratory settings (Cook M 1985[6], Knapska E 2010[7], Atsak P 2001[8], Jeon D 2010[9], Kim E 2010[10], Chen Q 2009[11], Olsson A 2007[12]), hampering the search for its underlying mechanisms. We developed a behavioral paradigm to study transmission of fear in rats, a social species widely used as a model system in Neuroscience, to examine the cues that mediate this process. Confirming previous studies (Atsak P 2011[8], Kim E 2010[10]), we found that observer rats freeze while witnessing a demonstrator cage-mate display fear responses, provided they had prior experience with an aversive shock. By systematically probing for the sensory cues that trigger transmission of fear, we found that observer rats respond to an auditory cue which signals the sudden immobility of the demonstrator rat – the cessation of the sound of motion. This study shows for the first time that the onset and offset of the sound of movement can be perceived by rats as a safety cue or danger signal, respectively. As freezing is a pervasive fear response in the animal kingdom (Mirza RS 2003[1], Siniscalchi M 2008[13], Blanchard RJ 1969[14], Forkman B 2007[15]) we believe that silence, or other signals of the sudden absence of motion, constitute a public cue that could be used by a variety of animals in the ecosystem to detect impeding danger.

INTRODUCTION

Most of what is known about the neural basis of fear was unraveled by studies using associative fear learning (reviewed in Maren S 2004[16]). Learning to fear cues that are associated with an experienced aversive event may be crucial, as these cues can be used to avoid future threats. However, many animal species are also able to use social cues to avoid threats (Mirza RS 2003[1], Ono M 2003[2], Wilson DR 2004[3], Platzen D 2005[4], Zuberbuhler K 2001[5]), a defense mechanism that may be less costly than learning from self-experience. Nonetheless, how the defense responses are triggered by social stimuli remains elusive. Recent studies have shown that rodents react to the distress of their cage-mates (Knapska E 2010[7], Atsak P 2001[8], Jeon D 2010[9], Kim E 2010[10], Chen Q 2009[11]) under laboratory settings, paving the way to the underpinning of the mechanism underlying fear transmission between animals. Our study aimed at examining the sensory cues that mediate transmission of fear between rats, as we believe these are crucial to guide the search for the neural basis of this process.

RESULTS

Social transmission of fear depends on observers' prior experience

In our experiments, a pair of cage-mate rats (one assigned to be the demonstrator and the other the observer) interacted in a two-partition chamber, which allowed rats to see, hear, smell and touch each other (see Methods and Fig. 1a). During the social interaction test (SI) we presented a tone cue, to which the demonstrator rat had previously been conditioned (conditioned demonstrator, CD). Thus, in this paradigm, the demonstrator rat displays fear in the absence of pain responses. Importantly, since previous reports showed that prior experience with foot-shocks is necessary for the ability to respond to the distress of the demonstrator (Atsak P 2011[8], Kim E 2010[10]), in our study, observer rats were assigned to one of two groups: 1) naïve observers (NO), that during prior experience were exposed to a conditioning chamber but no tones or shocks were delivered; 2) experienced observers (EO), which were exposed to the same conditioning chamber and received unsignaled footshocks (no tones were presented to these rats). In all experiments, we measured the time that demonstrator and observer rats spent freezing, a robust fear response (Blanchard RJ 1969[14]). Our results confirmed that experienced, but not naive, observer rats freeze upon the display of fear by their cage-mate (Fig. 1b and c) (Atsak P 2011[8], Kim E 2010[10]).

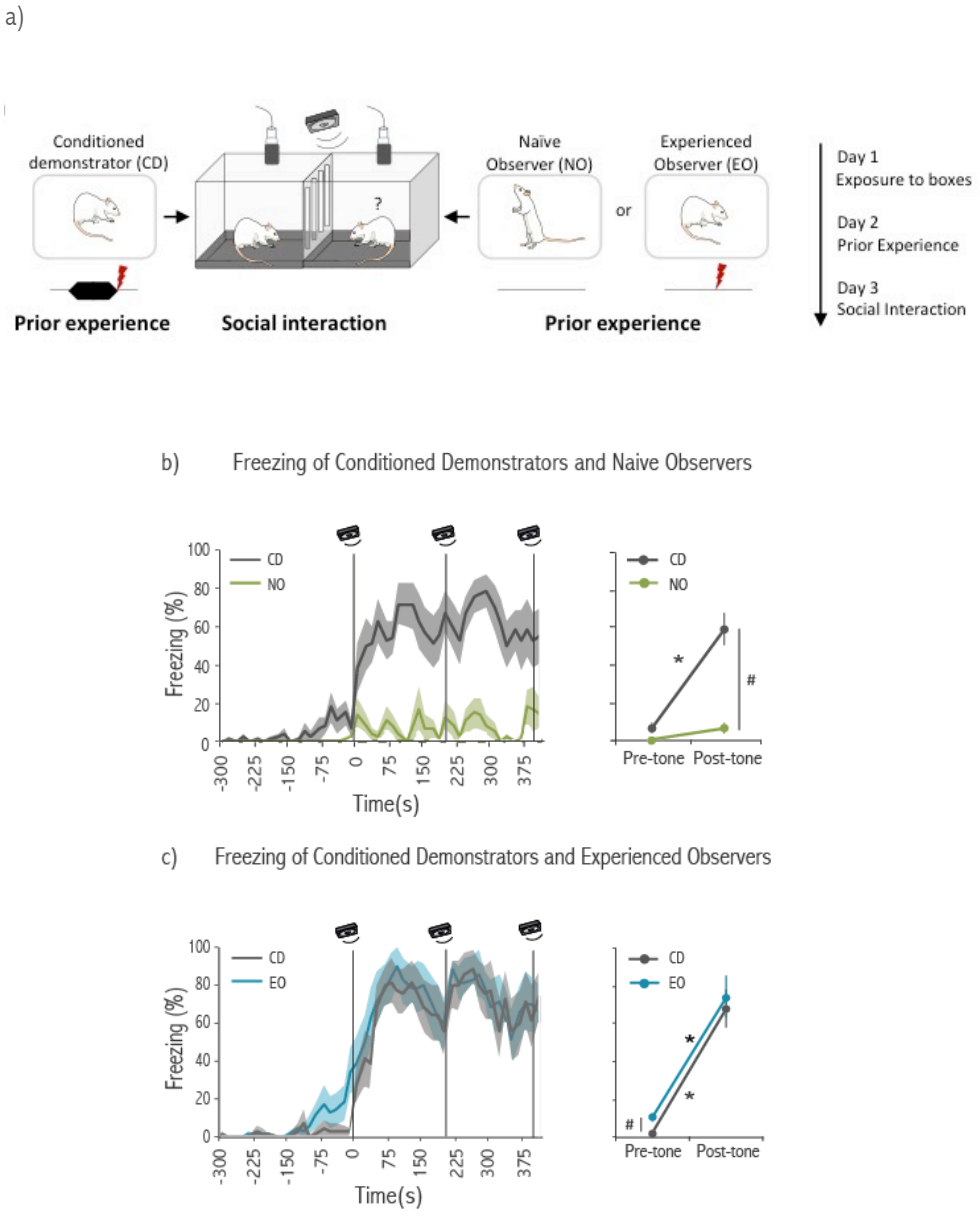


Figure 1 - Transmission of fear depends on the observers' prior experience. **a)** Schematic of the behavioral paradigm. **b)** Left panel, line graph showing mean±s.e.m. freezing of CD-NO pairs (n=8) over time during the SI test. Vertical black lines indicate time of each tone presentation. Right panel, line graph showing average freezing before and after the first tone presentation (Pre-tone and Post-tone) for demonstrators and observers (Pre- vs. Post-tone – demonstrators: V=0, p=0.024; observers: V=0, p=0.07; CD vs. NO Post-

tone: U=62, p=0.004). **c)** Same as **b)** for pairs CD-EO (n=8) (Pre- vs. Post-tone – demonstrators: V=0, p=0.031; observers: V=1, p=0.047; CD vs. EO Post-tone: U=28, p=0.721). * denotes $p<0.05$ for within animal comparisons and # denotes $p<0.05$ for comparisons between demonstrators and observers.

We also performed a set of experiments with demonstrators that were not conditioned to the tone (Naïve Demonstrators ND) in order to control if factors other than the defense responses of the demonstrators were significantly modulating the defense responses of the observers. In these experiments, NDs were paired with both EO and NO.

We didn't found any differences in freezing before and after tone in EO or ND in the EO-ND pairs (Pre vs. Post-tone - demonstrators: V=5, p=0.266; observers: V=8, p=0.293; ND vs. EO Post-tone: U=28, p=0.291). This result confirms that the display of defense responses in EO rats depends on the display of fear by the demonstrators and not on other factors, like the presentation of the 5KHz pure tone. Finally, when testing pairs of ND-NO we verified that these animals spend most of the time exploring the box, and that the presentation of the 5KHz didn't significantly change their behavior (Pre vs. Post-tone - demonstrators: V=13, p=1.009; observers: V=4, p=0.505; ND vs. NO Post-tone: U=12, p=0.280).

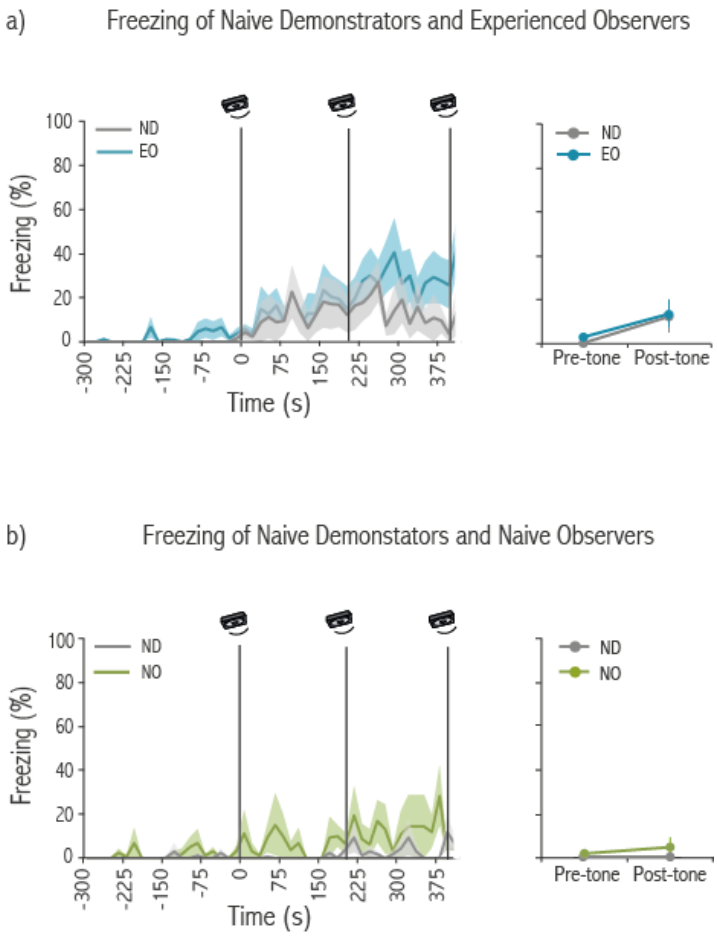


Figure 2 – Transmission of fear depends on the display of fear by demonstrators. **a)** Freezing during the SI test for pairs of ND-EO (n=9). **b)** Freezing during the SI test for pairs of ND-NO (n=7). (all graphs plotted as in Fig. 1).

Together, the previous results suggest that the display of defense behaviors by an observer animal depends on the display of fear by its partner and in its own prior experience.

Sensory cues in social transmission of fear

We then asked which cues or responses of the CD could trigger freezing in EO rats, starting by testing the role of intra-specific communication channels. Rats emit alarm calls (long vocalizations around 22kHz) when in distress (Portfors CV 2007[18], Blanchard RJ 1991[19], Choi SJ 2003[20]). However, their function is still a matter of debate (Blanchard RJ 1991[19], Choi SJ 2003[20], Woher M 2008[21]), particularly regarding whether they play a role in fear transmission (Atsak P 2011[8], Kim E 2010[10]). Thus, we recorded ultrasonic vocalizations (USVs) during the SI test. Although previous studies (Atsak P 2011[8], Kim EJ 2010[10]) show robust emission of alarm calls, in our experiments only one pair of the CD-NO and one pair of the CD-EO groups emitted this type of calls (see e.g. Fig. 3a). Thus, in our experimental conditions alarm calls do not mediate transmission of fear between rats. Interestingly, we found that all demonstrator-observer pairs emitted several affiliative calls (short calls above 35kHz typically observed during exploration and play (Portfors CV 2007[18], Parsana AJ 2012[22]) see e.g. Fig. 3b) before the first tone was played. However, in the pairs with conditioned demonstrators (CD-NO and CD-EO), these affiliative calls decreased dramatically after the first tone. This raises the possibility that affiliative calls may constitute a safety signal.

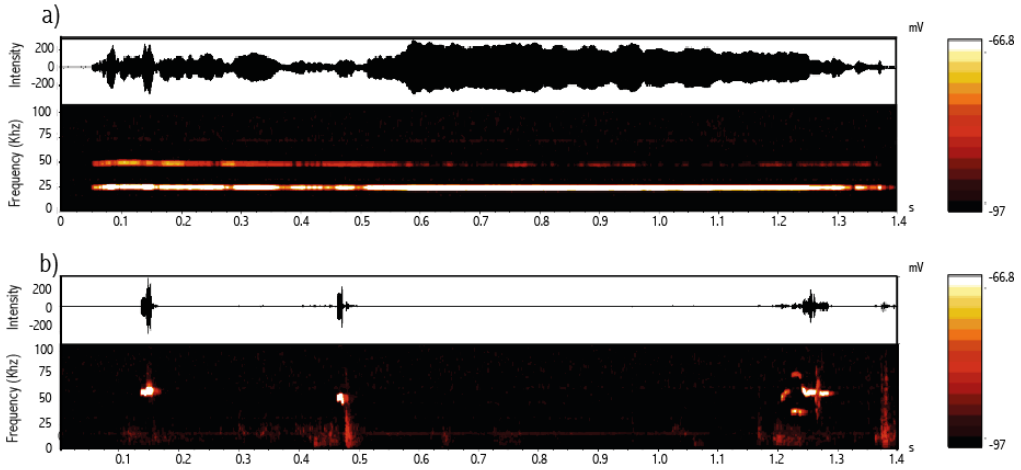


Figure 3 – Rats emit USVs of different types. **a)** Example alarm call. The alarm call shown was part of a train of calls emitted by the demonstrator rat after the first tone was played. In this spectrogram it is possible to see the harmonics of the fundamental frequency of the call. **b)** Example affiliative calls. Three affiliative calls emitted by the demonstrator rat can be seen (this sequence of calls was recorded during the initial period of exploratory behavior before the first tone was played). In both a) and b) the top panel shows the normalized sound envelop; the bottom panel shows sound spectrogram (intensity is color coded, scale bar on the right showing white as the lowest attenuation and black the highest attenuation, in dB).

Next, we examined the role of chemical communication since there is evidence that rodents exposed to pheromones released by stressed con-specifics display behavioral and autonomic responses indicative of stress (Kiyokawa, Y 2004[23], Brechbuehl, J 2009[24]). Even though alarm pheromones in rats are yet to be identified, there is evidence that these chemicals constitute short-range volatile signals (Inagaki, H 2009[25]). Thus, to test the role of these chemicals in the transmission of fear, we increased the separation between the rats during the SI test (by adding a 2nd partition – see methods). This manipulation blocked the access to both somatosensory and non-volatile

pheromones, while attenuating short-range chemical signals. Despite the separation between rats, there was a significant increase in freezing both in demonstrators and observers relative to baseline (Pre-tone vs. Post-tone demonstrators: V=0, p=0.016; observers: V=0, p=0.012 Fig. 4a). Furthermore, freezing after the first tone was not significantly different between demonstrators and observers (CD vs. EO Post-tone: U=63, p=0.1). This result suggests that contact, non-volatiles and possibly short-range chemical signals are not necessary to trigger fear in observer rats.

Since in our experiments the classical intra-specific channels of communication do not seem to be crucial for the transmission of fear between rats, we examined whether observer rats could be detecting a change in the behavior of the demonstrator, such as the onset of freezing (Blanchard RJ 1969[19]). Since immobility could be detected through visual cues, we performed the SI test in the dark (Fig. 4b). Again, we found that both demonstrators and observers showed a strong increase in freezing upon the presentation of the first tone (Pre-tone vs. Post-tone demonstrators: V=0, p=0.012; observers: V=0, p=0.008). Even though observers showed robust freezing after the first tone presentation, we found a small but significant decrement in freezing relative to the degree of freezing displayed by demonstrators (CD vs. EO post-tone: U=73, p=0.012). Hence, visual cues are not necessary for observer rats to respond to the fear of the demonstrator, although they may play a modulatory role.

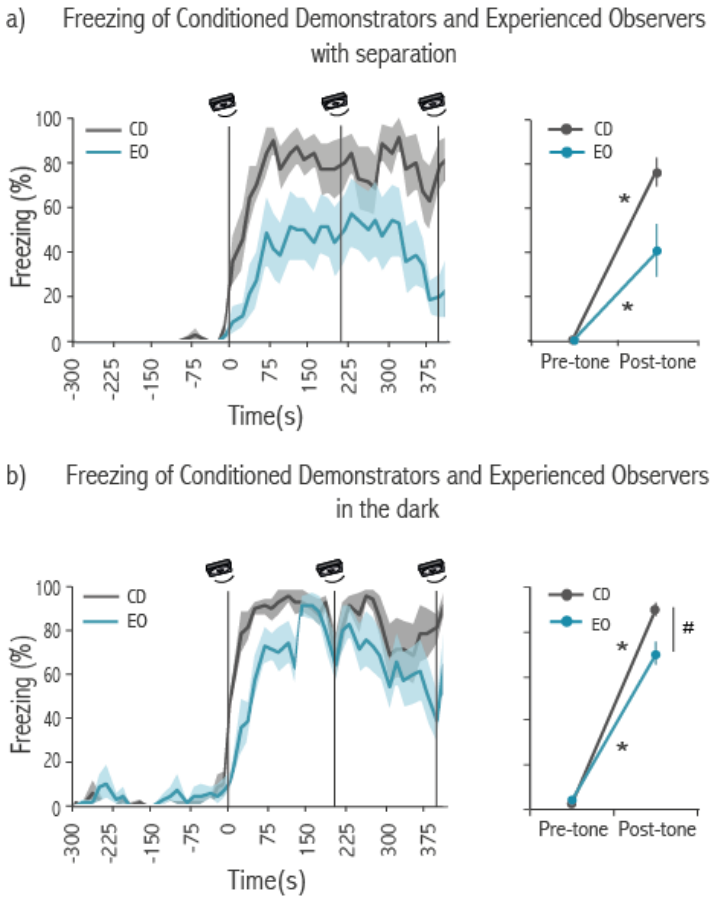


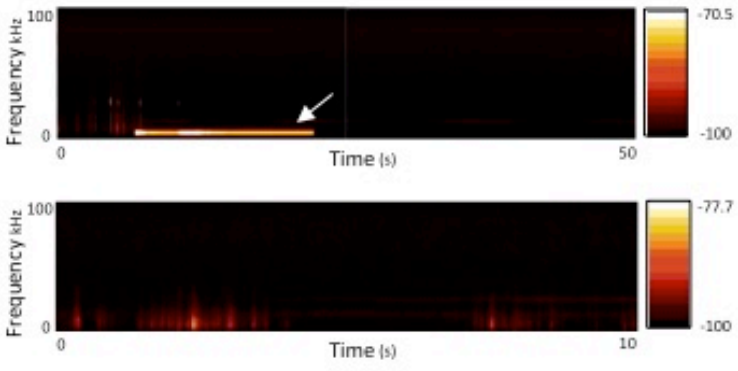
Figure 4 - Transmission of fear is independent of contact and visual cues. **a)** Freezing during the SI test with two partitions, blocking contact between demonstrator and observer rats (n=9). **b)** Freezing during the social interaction test performed in the dark (n=9) (all graphs plotted as in Fig. 1). * denotes $p<0.05$ for within animal comparisons and # denotes $p<0.05$ for comparisons between demonstrators and observers.

As visual cues are not crucial in triggering freezing in observer rats, we hypothesized that immobility of the demonstrator could be detected through the lack of movement-evoked sounds. Indeed, during the baseline period rats moved around in the social interaction chamber producing rustling sounds, which decreased dramatically when the demonstrator rats started freezing (see sound spectrogram in Fig. 5a). To test this hypothesis, during the SI test we

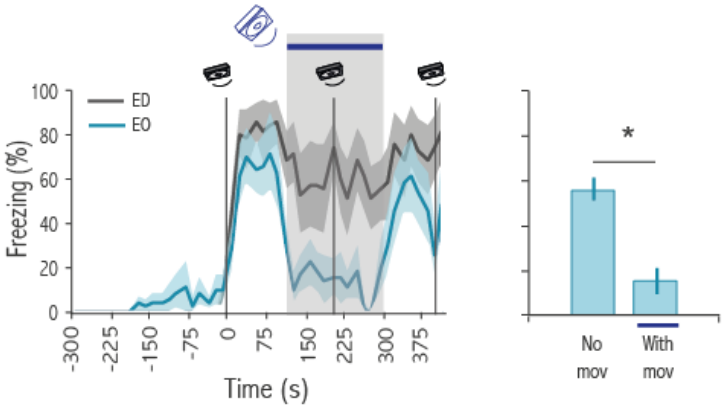
triggered freezing in CD-EO pairs by presenting the tone, but 90s later (when both rats were freezing) we played the sound recorded from a rat exploring the box for three minutes. If the lack of movement-evoked sounds, i.e. silence, is the cue that mediates freezing in observers, then playing the sound of the rat moving around should abolish their freezing. We found that playing the sound of a rat moving disrupted freezing by observers. Importantly, freezing resumed immediately after the sound playback, that is, silence re-instated freezing ($EO_{(CD-EO)}$ during 180s of movement-evoked sound playback vs. 180s with no playback (90s before and 90s after movement-evoked sound playback averaged together): V=28, p=0.032, Fig. 5b). In addition, freezing by the demonstrators remained unaffected by the sound playback ($CD_{(CD-EO)}$: V=23, p=0.151), suggesting that during this period other cues could signal the distress of the demonstrator. These cues were however not sufficient to drive freezing in observers.

In order to test if the decrease in observers freezing was due to the playback of the movement-evoked sound or to the disturbance in the acoustic scene, we repeated the previous experiment but instead played a 2Khz tone pips sequence with the same duration. We found that playing this sound didn't affect the freezing displayed by the observers ($EO_{(CD-EO)}$ during 180s of 2Khz pips playback vs. 180s with no playback (90s before and 90s after 2Khz pips playback averaged together): V=30, p=0.107 Fig. 5c). However, it should be noted that the playback of the 2Khz significantly increased the freezing of the CD ($CD_{(CD-EO)}$: V=36, p=0.028), probably due to some generalization to the 5KhZ tone to which they were previously conditioned. Therefore, we cannot exclude that this increase could contribute to the non-disruption of freezing in EO. However, it also suggests that not any acoustic disturbance is sufficient to significantly decrease freezing in EOs.

a) Sound spectrogram of movement-evoked sound



b) Freezing of Conditioned Demonstrators and Experienced Observers with playback of movement-evoked sound



c) Freezing of Conditioned Demonstrators and Experienced Observers with playback of 2Khz pips

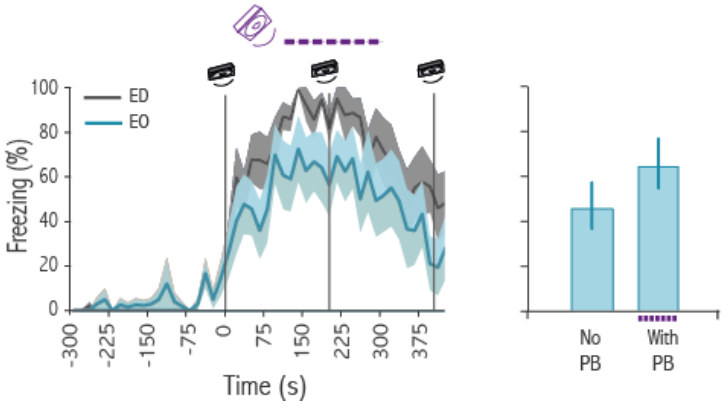


Figure 5 – Playback of movement-evoked sound disrupts freezing in EO. **a)** Top, example sound spectrogram from the time around the first tone presentation, showing the transition from sound to silence. Arrow indicates the pure tone. Bottom, sound spectrogram of a representative segment of the sound used in the playback experiment. **b)** Left panel, freezing during the SI test where grey box indicates time of movement-evoked sound playback (n=7). Right panel, bar graph showing freezing in the absence or presence of movement-evoked sound playback (indicated by blue horizontal line). **c)** Same as b) but purple dotted line indicates time of 2Khz tone pips playback (n=7) (all graphs plotted as in Fig. 1). * denotes $p<0.05$.

Together, these results suggest that the sound of movement is indicative of safety and that its sudden cessation is perceived as threatening. In addition, the recorded sound of movement did not include affiliative calls. Hence, although these calls might signal positive states in other rats, the sound of movement alone is sufficient to signal safety.

Finally, we tested whether the cessation of movement-evoked sound was sufficient to trigger freezing in experienced rats. To this end, we placed experienced or naïve rats alone in the social interaction chamber. During the test session the same movement-evoked sound used in the previous experiment was played continuously, except for two one-minute periods of silence (see Methods). Experienced, but not naïve rats froze during the periods of silence (Pre-silence vs. Silence $naive_{(mov\ sound)}$ rats: V=0, p=0.061; $experienced_{(mov\ sound)}$ rats: V=0, p=0.008, Fig. 6a and 6c) consistent with our finding that only experienced observers freeze in response to the fear displayed by the demonstrator rat. Hence, the absence of movement-evoked

sound, i.e. the onset of silence was sufficient to trigger freezing in experienced rats. Two alternative possibilities could explain this finding. On the one hand, silence may be aversive per se thereby triggering freezing. On the other hand, it may be the sudden offset of the movement-evoked sound that signals danger and triggers freezing. To disentangle these two possibilities, we performed the same experiment as above with experienced rats and added an arbitrary sound that filled the silence gaps (a continuous train of pure tone pips for the duration of the entire test session was delivered through a second speaker). With this manipulation we maintained the sudden offset of the motion cue but eliminated long periods of absolute silence. Experienced rats froze upon the cessation of the auditory motion cue even though there was the sound of the pure tone pips (Pre-silence vs. Silence experienced$_{(\text{mov sound + pips})}$ rats: $V=0$, $p=0.016$, Fig. 6b and 6c). Moreover, when comparing freezing during periods of silence, both experienced rats with and without trains of pure tone pips froze significantly more than naive rats (experienced$_{(\text{mov sound})}$ $>$ naive$_{(\text{mov sound})}$: $U=2$, $p=0.003$; experienced$_{(\text{mov sound + pips})}$ $>$ naive$_{(\text{mov sound})}$: $U=6.5$, $p=0.036$, no further differences were found). Together, these experiments show that the cessation of movement-evoked sound, rather than absolute silence, is sufficient to trigger freezing in rats that had prior experience with shock.

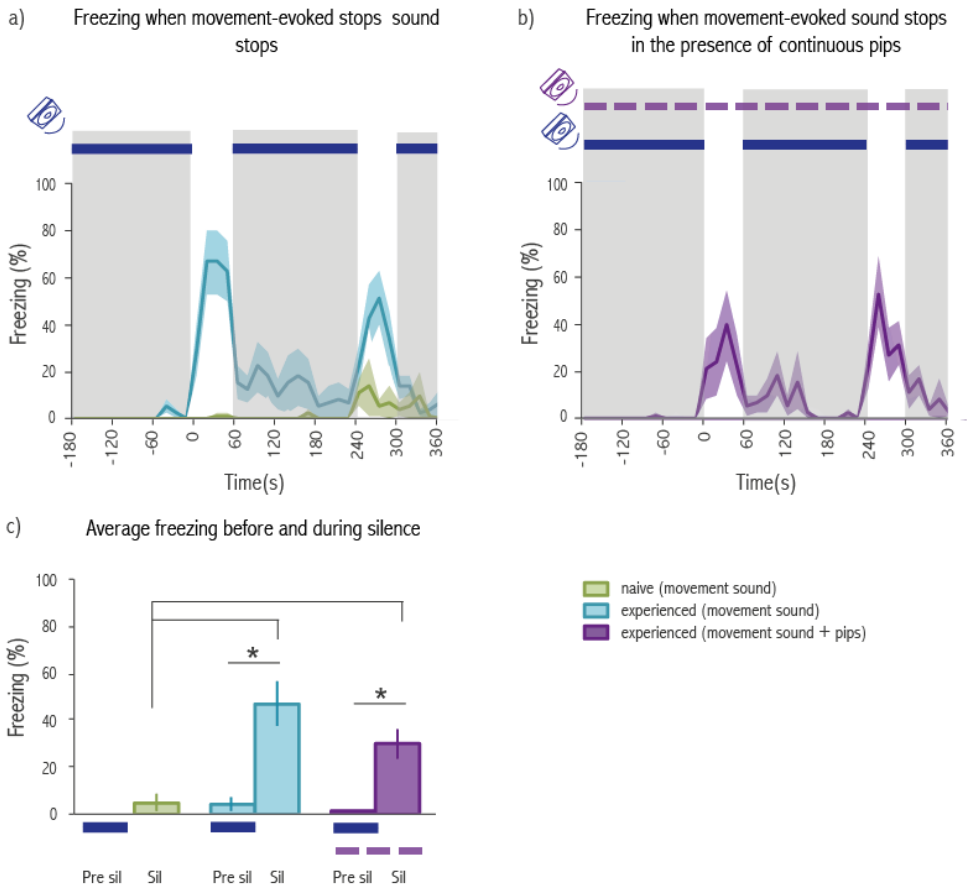


Figure 6 – Cessation of auditory motion signals triggers frezing in experienced rats. **a)** Line graph showing mean±s.e.m of freezing of experienced and naive rats throughout the test session. Grey boxes with blue horizontal bar shows time of playback of movement-evoked sound. **b)** Line graph showing mean±s.e.m of freezing of experienced rats. Grey boxes with blue horizontal line shows time of playback of movement-evoked sound and purple dashed line indicates playback of pure tone pips. **c)** Bar graph showing mean±s.e.m of percent time freezing during movement-evoked sound playback (indicated by blue horizontal line under bar graph) and during silence for naïve, experienced rats and experienced rats exposed to continuous tone pips (indicated by purple dashed line) * denotes $p<0.05$.

DISCUSSION

In conclusion, we show that in our experimental conditions observer rats did not rely on contact with the demonstrator, visual cues or alarm calls to detect fear. Instead, they use changes in auditory cues in the environment that are likely to signal the sudden transition from motion to immobility. We found that the absence of movement-evoked sound was necessary and sufficient to induce fear in observer rats. Previous studies have implicated visual cues (Jeon D 2010[9]) and alarm calls (Kim EJ 2010[10] but see Atsak P 2011[8]) in social transmission of fear. Thus, it is likely that a multitude of cues can be used by animals to detect fear in others, and that the context determines which cues will mediate this process. Furthermore, we replicated previous findings showing that rats freeze in response to the display of fear by their cage-mates, and that this response depends on prior experience with aversive footshocks. Future experiments are necessary to determine how exposure to the aversive shocks facilitates the response of rats to the display of fear by their cage-mate.

Several species use auditory cues to detect the presence of a predator either directly or indirectly through the defense responses of other individuals. These auditory cues range from the sounds of predators (Zuberbuhler, K 2001[5], Wilson M 2011[26]) to the alarm calls (Wilson DR 2004[3], Zuberbuhler, K 2001[5]) or auditory motions signals produced by escape behaviors of con-specifics (Hingee M 2009[27], Randall JA 2011[28]). In addition, there is evidence of heterospecific use of alarm signals (Zuberbuhler, K 2001[5]), including eavesdropping on alarm calls of other species (Magrath RD 2011[29], Ito R 2012[30]). Thus, animals learn to use a multitude of species-specific auditory signals, both from their own and from other species, to detect danger. In this study, we found that rats use the cessation of movement-evoked sound to detect freezing by another rat. As freezing is one of the most pervasive fear

responses being present in a wide range of vertebrate species (Mirza RS 2003[1], Siniscalchi M 2008[13] Blanchard RJ 1969[14], Forkman B 200715) we believe that silence constitutes a true public cue that rapidly spreads across animals within an ecosystem. Finally, this study will contribute to the current understanding of the neural mechanisms of fear, by providing a paradigm to study fear evoked by natural sounds. This may be particularly relevant in light of the fact that most of what we know about the neural mechanism of auditory perception and sound guided behaviors stems from studies using artificial sounds, which are likely to be processed very differently by the brain.

MATERIALS AND METHODS

Subjects

Naïve male Sprague Dawley rats (300–350 g) were obtained from a commercial supplier (Harlan). After arrival animals were pair housed in Plexiglas top filtered cages and maintained on a 12 h light/dark cycle (lights off at 8:00 p.m.) with *ad libitum* access to food and water. Rats were acclimated for at least one week before experimental manipulation and all animals were handled for a few days before each experiment. All behavioral procedures were performed during the light phase of the cycle. The Instituto Gulbenkian Ciência and Champalimaud Foundation follows the European Guidelines of animal care. The use of vertebrate animals in research in Portugal complies with the European Directive 86/609/EEC of the European Council.

Behavioral Apparatus

Two distinct environments were used in this study, the conditioning chamber and the social interaction box, which were located in the same procedure room. The conditioning chamber (model H10-11R-TC, Coulbourn Instruments), had a shock floor of metal bars (model H10-11R-TC-SF, Coulbourn Instruments) and was inside a high sound isolation chamber (Action automation and controls, Inc). In this chamber a precision programmable shocker (model H13-16, Coulbourn Instruments) delivered the foot-shocks and the tones were produced by a sound generator (RM1, Tucker-Davis Technologies) and delivered through a horn tweeter (model TL16H8OHM, VISATON). The sound was calibrated using a Brüel and Kjaer microphone (type 4189) and sound analyzer (hand held analyzer type 2250). The social interaction box consisted of a two partition chamber made of clear Plexiglas walls (60cm wide x 34cm height x 27cm depth) (Gravoplot). The chamber was

divided in two equal halves by a clear Plexiglas wall with 0.7cm wide vertical slits separated by 1.5cm, which allowed rats so see, hear, smell and touch each other. On each side of the chamber the floor contained a tray with bedding. The social interaction box was placed inside a sound attenuation chamber (80cm wide x 52.5cm height x 56.5 cm depth) made of MDF lined with high-density sound attenuating foam (MGO Borrachas Técnicas) and a layer of rubber. Although the two chambers were quite distinct, to minimize generalization between the two environments when the social interaction took place in the dark conditioning took place in the light and vice versa. In addition, the boxes were cleaned with two cleaning fluids with distinct odors. The rats' behavior was tracked by a video camera mounted on the ceiling of the attenuating cubicle in the case of the conditioning chamber, and by two cameras mounted on the back wall of the attenuation box (one behind each partition) in the case of the social interaction box. A surveillance video acquisition system was used to record and store all videos on hard disk and freezing behavior was automatically scored using FreezeScan from Clever Sys. Ultrasonic vocalizations were recorded in the social interaction chamber through two microphones, placed over each of the two partitions (Avisoft-UltraSoundGate system 416H recorded with microphones model CM16/CMPA).

Behavioral procedures

All experiments were done with pairs of cage-mate rats, one rat was the demonstrator and the other the observer. All rats were exposed to the two environments for fifteen minutes on the first day.

Social Transmission of Fear experiments

On the second day, rats were place in the conditioning chamber and subjected to different protocols corresponding to the different conditions of prior experience:

Conditioned demonstrators (CD): after an initial five minute period, rats received five tone-shock pairings (tone: 20s, 5 kHz, 70dB; shock 1mA, 0.5s), such that tone and shock co-terminated, with an average intertrial interval of 180s.

Naïve demonstrators (ND): rats were placed in the conditioning chamber and received five tone presentations (same tones with the same schedule as for CD rats). Presentation of the tones to the naïve demonstrators habituated these animals to the novel stimulus insuring no freezing to the tone during the social interaction test.

Experienced observers (EO): rats were placed in the conditioning chamber and received five shock presentations (same shock with the same schedule as for conditioned demonstrator).

Naïve observers (NO): rats were placed in the conditioning chamber for the same length of time as all other animals but received no tones or shocks.

On the third day, the different pairs of rats (resulting from all combinations of the different prior experience protocols: CD-NO, CD-EO, ND-EO, ND-NO) were tested in the social interaction box. Each rat, the demonstrator and the observer, was placed in one side of the two-partition box (side varied randomly across different pairs of rats). After a five minute baseline period, the three tones (same tone as above) were presented, with a three minute inter-trial interval.

To test the role of contact, non-volatiles and short range volatile chemicals the same procedure as above was used for CD-EO pairs. However, instead of a single wall dividing the two partitions, two identical walls placed 12cm apart separated the two rats.

To test the role of visual cues, CD-EO pairs were used but the social interaction test was performed in complete darkness.

To test the role of auditory motion cues a single rat was recorded as it moved around in one of the partitions of the social interaction box with bedding on the floor. The sound of this rat moving around was recorded using the Avisoft system. The recorded sound was used for the playback experiments.

Playback during social interaction

CD-EO pairs followed the same procedure as before, with the social interaction taking place in the dark to avoid conflicting evidence between visual and auditory cues. However, during the social interaction session 90s after the first tone presentation the recorded sound of a rat moving around was played back from a second speaker for three minutes.

The same procedure was followed for the playback of the 2KHz tone pip sequence (2kHz, 55dB pips of 250ms, at 0.67 Hz).

Playback to single rats

Naive (exposed to the conditioning box for 15 minutes) or experienced (initial five minute period followed by 3 shocks, 1mA, 0.5s, ITI 180s) rats were placed in one of the partitions of the social interaction box alone with a ball (to minimize generalization). In the first experiment, the recorded sound of

movement was played throughout the test session (10 minutes), except during two one-minute periods of silence. In a control experiment, experienced rats were exposed to the same procedure but an additional sound (2kHz, 55dB pips of 250ms, at 0.67 Hz) was continuously played through a second speaker.

Statistical Analysis

As our variables are not normally distributed and sample sizes are small, we used non-parametric tests only. For our statistical analysis on the social transmission of fear experiments, we focused on the three minutes after the first tone and used as baseline the three minute preceding it. In this manner we ensure that both measures have the same sampling time.

For the experiments of the movement-evoked sound playback during the social interaction (figure 1D), freezing was averaged during the 180s of playback and compared with periods of no playback of the same total length (average of 90s before and after the playback). For the experiments with the rats alone, freezing was averaged over the two minutes of silence and compared with the average freezing over the two one minute periods preceding the silence (during which the movement-evoked sound were still being played).

In all experiments, for comparisons within animals we used Wilcoxon Signed-Rank tests and for comparisons between animals we used Mann-Whitney tests. All comparisons were two-tailed. When multiple comparisons were made the reported p values were corrected using a sequential Bonferroni method. All the data was presented as average $\pm$ s.e.m.

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CHAPTER 3 - Neuronal Pathways underlying Defense Responses triggered by the cessation of movement-evoked sounds

"O Silêncio é o combustivel do medo"

Unknown street artist, S. Sebastião da Pedreira, 2015

Acknowledgments

Marta Moita and Ana Pereira designed the experiments in this chapter with the help of Susana Lima and Andreia Cruz. Ana Pereira performed all the experiments and analyzed the data. Ana Pereira, Marta Moita, Susana Lima and Andreia Cruz discussed results.

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ABSTRACT

Several species use social information to detect impending danger (Seyfarth RM 1980[1], Mirza RS 2003[2], Wilson DR 2004[3]). We previously developed a behavioral paradigm to study social transmission of fear, and found that rats perceive the cessation of movement-evoked sound produced by conspecifics as a signal of danger and its resumption as a signal of safety (Pereira AG 2012[4]). The Lateral Amygdala is a structure widely implicated in fear responses (Maren S 2004[5], Pape H 2010[6], Herry C 2014[7]), receiving auditory inputs from both cortical and thalamic pathways. Therefore, we hypothesize that the cessation of the movement-evoked sound leads to the activation of auditory inputs to the Lateral Amygdala switching on freezing. In order to test this hypothesis, we exposed rats to the sound of another rat moving around with periods of silence, during which rats normally freeze. We optogenetically inactivated the Lateral Amygdala during the period of silence and found that this manipulation disrupted freezing. To further characterize the neuronal pathways underlying this behavior, we compared *c-fos* expression in different sub-regions of the auditory thalamus of animals exposed to the sound interrupted by silence to that of animals exposed to continuous sound. We found a significant increase in the number of *c-fos* expressing cells in the dorsal Medial Geniculate Nucleus of animals exposed to the sound with silence periods. The dorsal Medial Geniculate Nucleus has been shown to have a higher proportion of cells responding to the offset of sounds compared to other nuclei in the auditory thalamus (Bordi F 1993[8]). These responses may signal the cessation of the movement-evoked sound, driving activity in the Lateral Amygdala. This study contributes to our current understanding of the neural mechanisms of fear, by providing information on how fear can be regulated by natural sounds.

INTRODUCTION

When an animal is in the presence of a threat, both behavioral and physiological changes occur that promote the avoidance of the menace. These changes can be alterations in heart rate and respiration, display of defense behaviors, release of chemical signals, amongst others. Individuals in the surroundings of the fearful animal (both con and heterospecifics) may detect some of these changes, that become cues that signal an impeding danger. The detection of such cues can therefore trigger defense behaviors in observers, in a phenomenon called Social Transmission of Fear (STF).

The ability to use information conveyed by others has been reported in many species (Chen F 2012[9], Mirza RS 2003[2], Ito R 2009[10], Wilson DR 2004[3], Mateo JM 1996[11], Hingee M 2009[12], Seyfarth RM 1980[1], Olsson A 2007[13]) and can bring both immediate and long term benefits (Griffin AS 2004[14]). Currently, there is a vast literature concerning the neuronal mechanisms underlying the display of defense behaviors when an individual directly detects a threat (reviewed in Gross C 2012[15], Herry C 2014[7]). However, very little is known about the circuits involved in fear responses when this detection occurs indirectly through information conveyed by others. In order to address this question, others and we previously developed a behavioral paradigm to study STF under laboratory settings, using rodents as an experimental model (Pereira AG 2012[4], Atsak P 2011[16], Kim EJ 2010 [17], Jeon D 2010 [18], Sanders J 2013 [19]). In our previous study we found that observer rats freeze while witnessing a demonstrator cage-mate displaying fear responses, provided they had prior experience with an aversive shock. By probing for different sensory modalities, we found that observer rats respond to an auditory cue that signals the sudden immobility of the demonstrator rat – the cessation of the sound of motion.

Most of what is know about acoustically driven defense behaviors was unrevealed by Auditory Fear Conditioning (AFC) studies where an initially neutral auditory stimulus (conditioned stimulus, CS) occurs in conjunction with an innately aversive stimulus (Unconditioned Stimulus, US). After such pairing, the presentation of the CS triggers defense behaviors in trained subjects. These studies demonstrated that the Lateral Amygdala (LA) is necessary for learning, storage and expression of defense behaviors triggered by sound (reviewed in Maren S[5], Pape H 2010[6], Herry C 2014[7]). However, most of these studies used artificial sounds that are not part of the rats' environment. Knowledge about how ethologically relevant sounds trigger defense behaviors in rodents is quite sparse, being mostly limited to alarm calls (Parsana AJ 2012[20], Parsana AJ 2011[21], Sadananda M 2008[22], Furtak S 2007[23]). One study in particular found that cells in amydala show tonic and phasic excitatory responses to the presentation of alarm calls (Parsana AJ 2011[21]). However, it has not been investigated if activity in LA is necessary for the display of defense behaviors triggered by this auditory stimulus (Parsana AJ 2012[20]). Interestingly, it has been recently reported that innate flight behavior induced by a broadband loud sound is mediated by corticofugal neurons in the auditory cortex targeting the inferior culliculus (Xiong XR 2015[24]). This study suggests that other pathways independent of the amygdaloid nucleus underlie defense behaviors triggered by sound. Importantly, in the mentioned studies the defense behaviors were triggered by the presentation of an auditory cue. However, in our paradigm freezing is triggered by the termination of a sound. The differences between our stimulus and the ones previously used to look at auditory driven defensive behaviors motivated us to investigate whether activity in LA is necessary for the display of fear triggered by the cessation of the sound of movement. In order to address this question we exposed rats to the

recorded sound of movement with a silent gap and optogenetically inactivated the LA during this gap.

Finally, we assessed putative auditory inputs to LA. Previous electrophysiology studies performed in anesthetized rodents found cells in the Medial Geniculate Body that respond to the offset of sounds (Bordi F 1993[8], He J 2001[25]), and several of its subnuclei send direct projections to the LA (Doron NN 1999[26], Namura S 1997[27]). To further characterize the neuronal pathways involved in the detection of the cessation of the movement-evoked sound, we compared *c-fos* expression in different sub-regions of the auditory thalamus of animals exposed to the sound interrupted by silence to that of animals exposed to continuous sound.

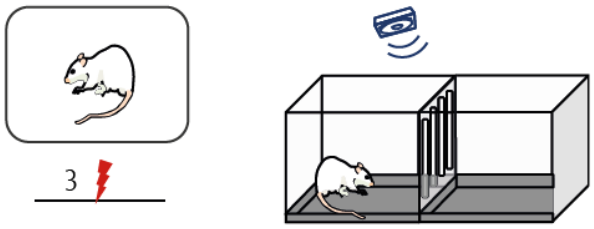
With the present study we hope to gain insight into the neuronal pathways involved in the display of defense behaviors triggered by an ethologically relevant auditory stimulus.

RESULTS

Optogenetic inhibition of LA during the cessation of the movement-evoked sounds

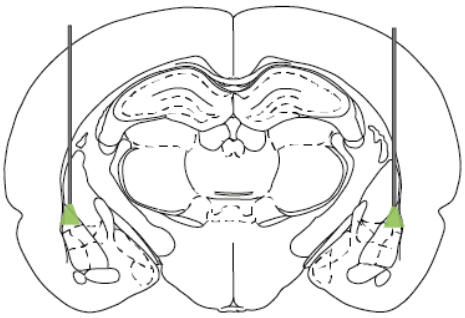
In our previous work we found that the cessation of a movement-evoked sound is sufficient to trigger freezing in rats that had prior experience with footshocks. We recapitulated this experiment in order to test if activity in LA is necessary for the display of freezing during the silence inserts. We exposed rats to two different environments, a conditioning box and a test box . Rats received three unsignaled footshocks in the conditioning box and the following day were tested for their response to the cessation of the movement-evoked sound in the test box (see Fig. 1a and Methods). The test consisted in the playback of a previously recorded sound of a naïve animal moving in a tray with bedding (movement-evoked sound) with a period of silence that lasted one minute. To test for the necessity of neuronal activity in LA in this response, we used an optogenetic approach to inhibit neural activity starting just before the transition from movement-evoked sound to silence until the end of the silence period. Specifically, we infected LA neurons with an AAV5 virus encoding an Archaerhodopsin-T(ArchT)/GFP fusion protein under the control of the cytomegalovirus early enhancer/chicken β actin (CAG) promoter (AAV5-CAG-ArchT-GFP). ArchT is a green/yellow light-responsive outward proton pump and has been shown to potently inhibit neural activity when activated by light (Han X 2011[28]). To control for the effect of light delivery in the brain, we also tested a group of rats with fibbers implanted in LA receiving the same light stimulation but with no virus expression (Ctr for light) (Fig. 1b, c, and d)).

a)



Day 1 to 4 - Exposure Day 5 - Prior Experience Day 6 - Silence test

b)



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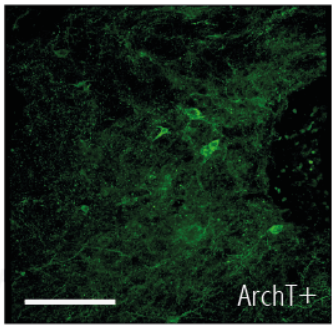

d)

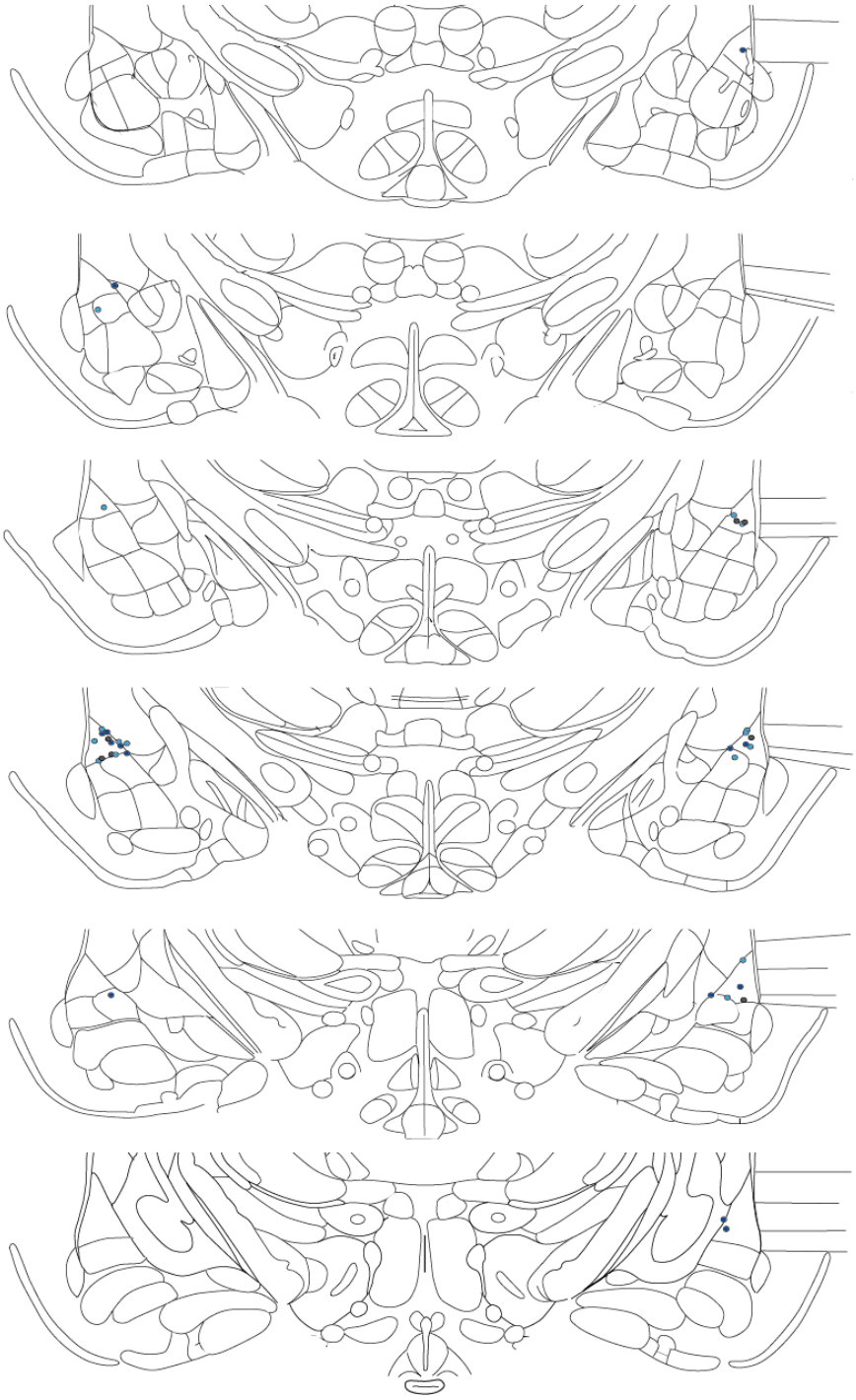

Figure 1 – Schematics of the behavioral paradigm and ArchT expression in LA. **a)** Schematics of the behavioral paradigm. **b)** Representative image of fibber implants and optogenetic illumination in LA. **c)** ArchT^{+} cells in LA. **d)** Coronal slices representing fiber placements for the ArchT group (dark blue), Ctr for light group (light blue) and Ctr for virus group (grey). Scale bar represents 100μm.

During the baseline period (one minute before silence onset) rats in both groups froze very little, and we found no difference between the average freezing of ArchT and control rats (ArchT vs. Ctr for light: U=34, p=0.502, Fig. 2a and b). However, during the silence period rats in the ArchT group froze significantly less than controls (ArchT vs. Crt for light: U=6, p=0.026, Fig. 2a and b). We also found a significant difference between the two groups when comparing levels of freezing during silence normalized to baseline freezing (ArchT vs. Crt for light: U=3, p=0.005, Fig. 2c). In addition, we found that whereas rats in the Ctrl for light group significantly increase freezing during the silence period, this increase was not significant in the ArchT group (Ctrl for light: V=0, p=0.014; ArchT: V=1; p=1.06, Fig. 2d). Together, these results suggest that activity in LA is necessary for the display of freezing triggered by the cessation of the movement-evoked sound.

Our control group, Ctr for light, showed that the absence of freezing triggered by silence in the ArchT group was likely due to the inactivation of LA cells and not to the presence of a fiber in LA or the delivery of light. However, in order to test if the expression of the ArchT/GFP fusion protein affects activity in LA per se independently of light, we tested a small group of animals (Ctr for virus) with AAV5-CAG-ArchT-GFP in the LA and implanted fibber optic, but without the delivery of light during the silence period (Fig. 2d grey plot). All

three rats increased their freezing from baseline to silence (difference in freezing was 37,93%, 43,13% and 67,80% for each individual rat) and the median change in the percent time freezing in this group was of 43,13% while in the ArchT was of 2,20%. These results suggests that the disruption of freezing evoked by the cessation of movement-evoked sound is due to the inactivation of LA cells and not due to a general compromise of LA function due to the expression of the fusion protein.

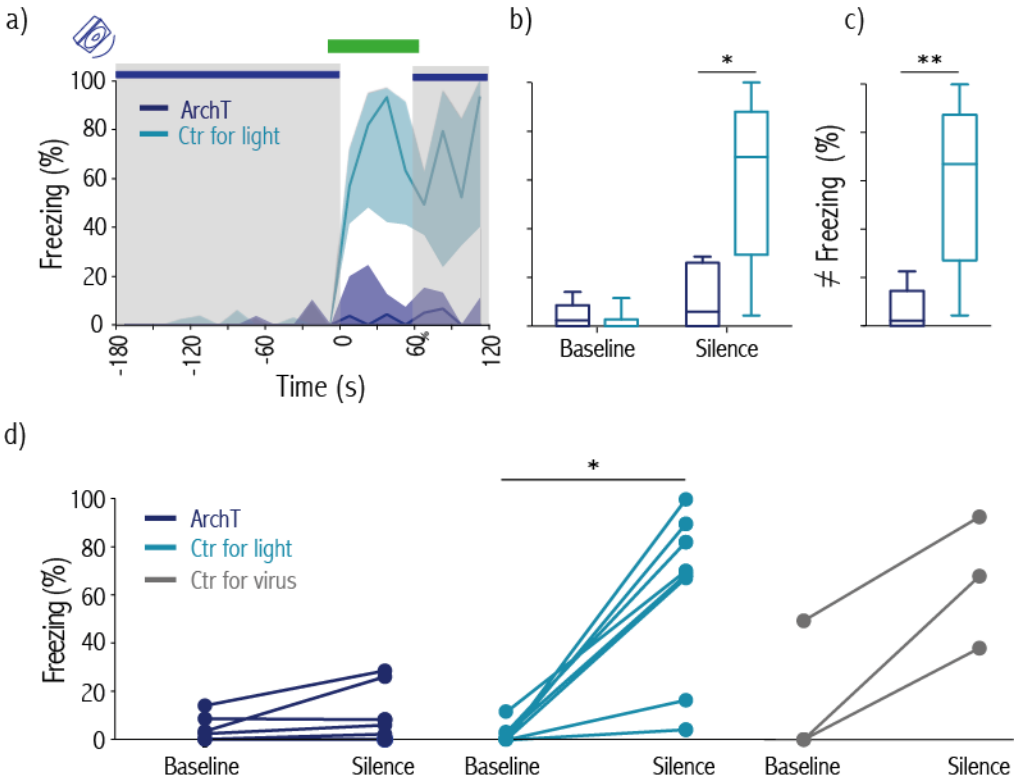

Figure 2 – Optopgenetic inactivation of the LA significantly decreases freezing triggered by the cessation of the movement-evoked sound **a)** Movement-evoked sound was presented (indicated by grey boxes) with one minute silence insert to rats in the ArchT (n=7) or Ctr for light groups (n=8). Green bar indicates the period during which 556nm light was on. Line graph shows median freezing throughout the test session (shaded area shows dispersion of the data given by the 1st and 3rd quartile). **b)** Box plot shows

freezing of rats in the ArchT and Ctr for light groups, during baseline and silence. Median, 1st and 3rd quartile are shown (error bars indicate minimum and maximum values). **c)** Same as **b)** but showing the difference in freezing between baseline and silence periods. **d)** Line graph showing average time spent freezing during baseline and silence periods for each individual of the ArchT, Ctr for light and Ctr for virus (n=3) groups. * denote $p < 0.05$ and ** denote $p < 0.01$.

c-fos expression in the Auditory Thalamus

In order to characterize the neuronal circuits underlying the present acoustically driven defense behavior, we focused our attention in the auditory thalamus, one of the first relay stations of auditory information in the brain. Importantly, it has been previously shown that different subnuclei of the medial geniculate body (MGB) of the auditory thalamus have direct projection to the LA (Doron NN 1999[26], Namura S 1997[27]). In addition, previous studies found cells that respond to the termination of sounds in this structure (Bordi F 1993[8], He J 2001[25]). To address if cells in the different subnuclei of the MGB respond to the cessation of the movement-evoked sound, we looked at *c-fos* expression as a marker of neuronal activity. We compared *c-fos* expression in the different sub-regions of the thalamus of rats exposed to the sound interrupted by silence (Silence group) to that of animals exposed to the playback of the sound (Continuous PB group). In this experiment we subjected the animals to two periods of silence, in order to increase likelihood of seeing activation of *c-fos*. Behavioral analysis shows that the insertion of silence gaps significantly increased freezing when compared to the periods of one minute preceding the silence (Pre Silence) in the Silence group (Pre Silence vs. "Silence": V=1, p=0.034, Fig. 3a). In the Continuous PB group we did not find

differences in freezing between equivalent periods (Pre Silence vs. "Silence": V=5, p=1.000, Fig. 3b. These results confirmed our previous finding that freezing is driven by the cessation of the movement-evoked-sound and not by other factors like generalization to the box (Fig. 3a and b).

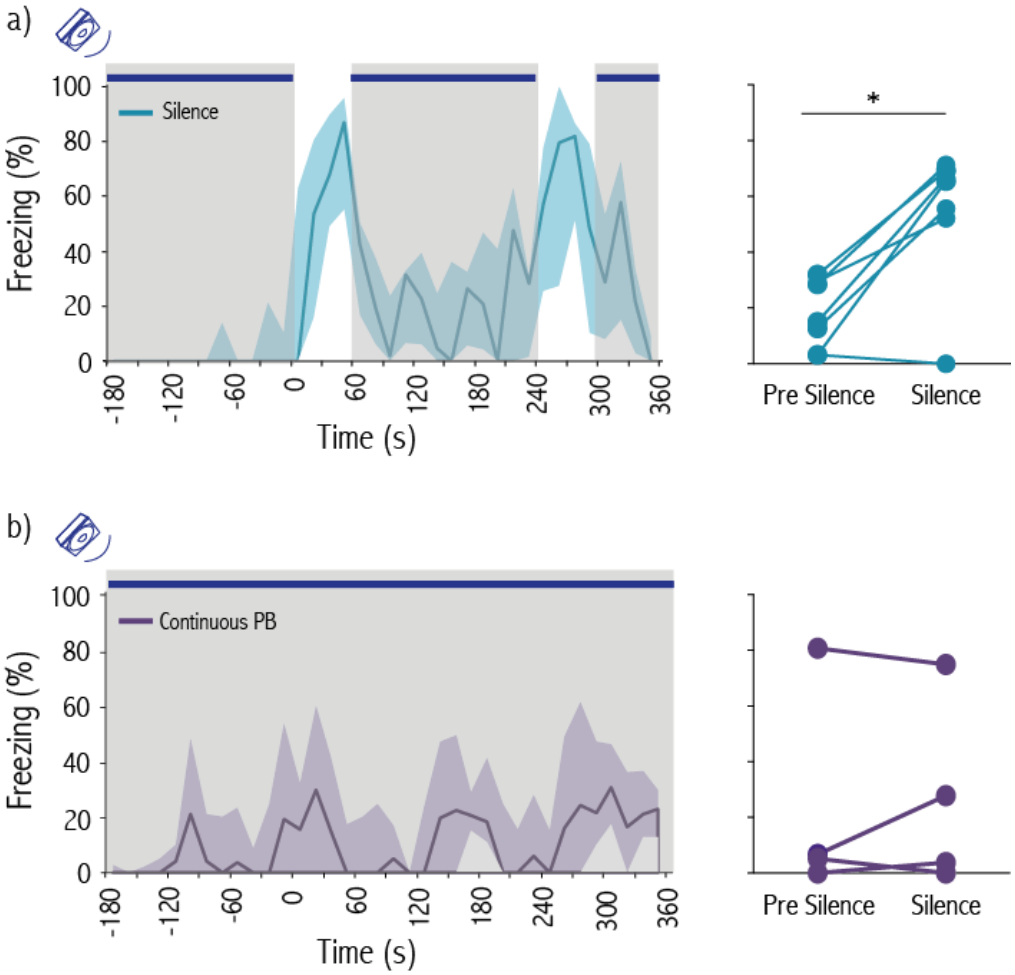

Figure 3 – Freezing is triggered by the cessation of the movement-evoked sound **a)** Left panel, line graph shows median freezing throughout the test session (shaded area shows dispersion of the data given by the 1st and 3rd quartile). Movement-evoked sound (indicated by grey boxes) was interrupted by two periods of one minute of silence (n=7). Right panel, line graph shows time spent freezing averaged across the two periods of one minute preceding each silence gap (Pre Silence) and averaged across the two periods of one

minute during silence (Silence) for each individual; **b)** Same as **a)** but for rats exposed to a continuous playback of the movement-evoked sound (n=4). * denote $p < 0.05$.

We quantified *c-fos* expression in four subnuclei of the auditory thalamus that project to the LA, namely the medial and dorsal division of the MGB (MGm and MGd, respectively), the suprageniculate nucleus (SG) and the posterior intralaminary nucleus (PIN). We only found a significant difference in the average number of *c-fos* labeled cells in the MGd (Silence vs Continuous PB: U=27, p=0.018), where there were an increased number of positive cells in the Silence group (Fig. 4a to d). In order to evaluate if these differences were homogeneous along the anterior-posterior axis we divided our brain sections in Anterior (ant.), Medial (med.) and Posterior (post.) regions (see methods) and tested for an effect of position along the anterior-posterior axis, exposure to silence and their interaction. A 2 group (Silence, Continuous PB) x 3 positions (ant., med., post.) mixed model ANOVA for *c-fos* expression indicated a significant effect of group ($F_{2,26} = 721.682$, $p<0.0001$), a significant effect of position ($F_{2,26} = 21.699$, $p<0.0001$) and a significant interaction between group and position ($F_{1,26} = 483.262$, $p<0.0001$). Further post-hoc analysis indicate a significant difference in *c-fos* expression between the posterior region of the silence group and the anterior and medial region of the same group (p=0.001 and p=0.014 respectively) and between the posterior region of the Continuous PB and Silence groups (p=0.009) (Fig. 4e). These results show that *c-fos* expression is different along the anterior-posterior axis in both groups, increasing from the anterior to the posterior region. The treatment to which the animals were exposed, whether the playback of the movement

evoked sound continuously or with silence inserts, influences such expression being the effect more marked in the posterior region.

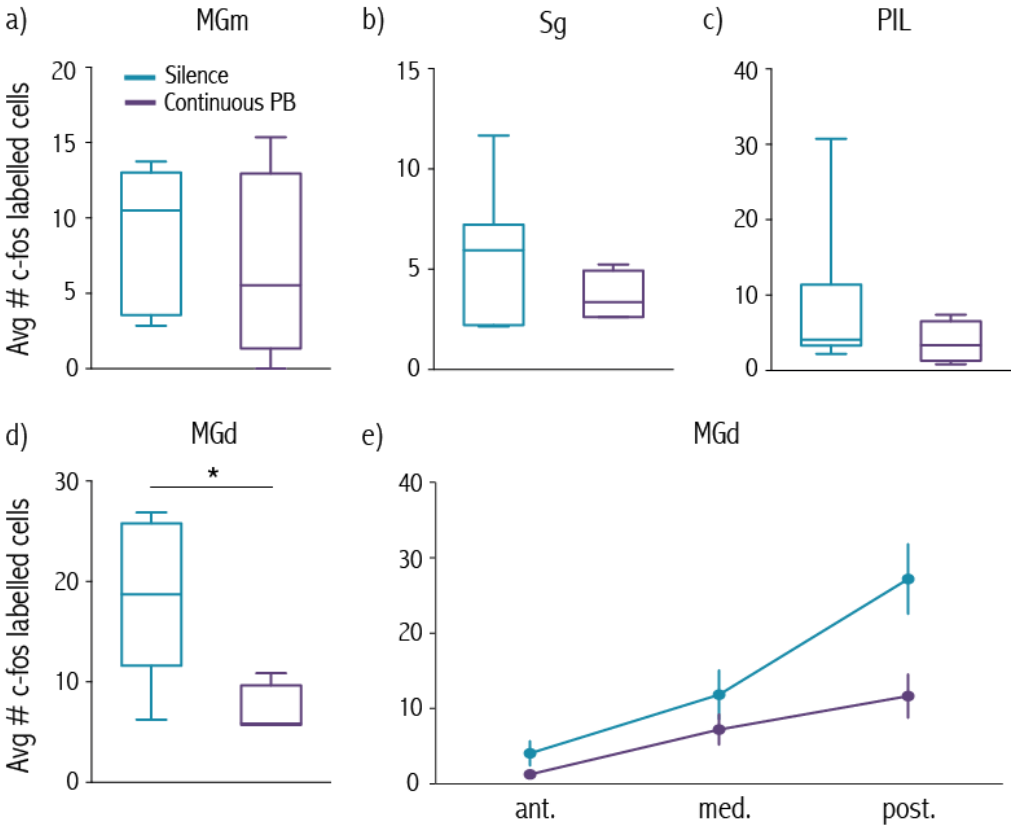

Figure 4 – Increased number of *c-fos* labeled cells in the MGd of rats exposed to the playback of the movement-evoked sound with Silence inserts **a)** Box plot shows average number of *c-fos* labeled cells in the MGm of rats exposed to the playback of the movement-evoked sound with (Silent) and without (Continuous PB) silent gaps. Median, 1st and 3rd quartiles are shown (error bars indicate minimum and maximum values). **b) c)** and **d)** same as **a)** but for Sg, PIL and MGd respectively. **e)** Line graph showing average number of *c-fos* labeled cells in the MGd along the anterior-posterior axis for both Silence and Continuous PB groups. In all graphs it is represented the average number of *c-fos* labelled cells per 10^5 μm^2. * denote $p < 0.05$.

Projections from the Auditory Thalamus to LA

Direct projections from different subnucleus of the thalamus to the LA have been previously reported (Doron NN 1999[26], Namura S 1997[27]). In order to confirm if cells in the MGd project to the region of the LA we inactivated in our task, we injected a retrograde neuronal tracer Cholera Toxin subunit B (CT-B) in LA using the same coordinates we used for viral injections (Fig. 5b). As previously reported (Doron NN 1999[26], Namura S 1997[27]), we found labeled cells in several subnucleus of the auditory thalamus namely the Sg, MGm and PIL (Fig. 5c). Importantly, we found labeled cells in MGd even in the more posterior sections where we detected more *c-fos* labeled cells in our previous experiments (Fig. 5c and higher magnification in 5d, e, and f).

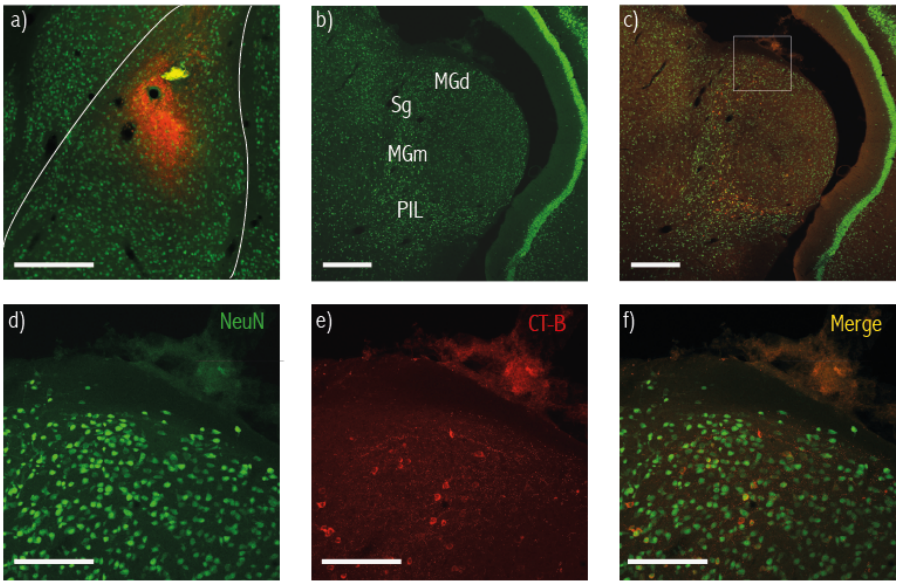

Figure 5 – Direct projections from the MGd subnucleus of the auditory thalamus to LA. Coronal sections of the rat brain showing **a)** representative image of injection site of retrograde tracer CT-B in LA. White lines delineate LA. **b)** representative image of different subnuclei of the auditory thalamus. **c)** CT-B labeling in different subregions of the auditory thalamus (image shows double labeling with NeuN and CT-B). Square indicates region of the MGd

shown in magnified images in **d) e)** and **f).** A representative photomicrograph of the MGd with neurons labeled with NeuN in green (**d**), showing cells labeled with CT-B in red (**e**), and overlay of the two images, with double-labeled neurons in yellow (**f**). Scale bar represents 100μm in **a) b)** and **c)** and 200 μm in **d) e)** and **f)**.

However, it should be noted that during the injections there was some spread of CT-B to the Caudate Putamen that also receives inputs from the auditory thalamus (Doron NN 1999[26]). Therefore, some of the labeled cells in the auditory thalamus may result from retrograde transport from this area and not from LA.

DISCUSSION

In our previous study (Pereira AG 2012[4]) we found that rats with prior aversive experience with footshocks freeze to the cessation of a movement evoked sound. Since naïve animals do not freeze to this cue, we are in the presence of a learned acoustically driven defense behavior. Given the vast literature showing that the LA is important for the learning, storage and expression of defense behaviors driven by sounds (Maren S 2004[5], Pape H 2010[6], Herry C 2014[7]), we tested the role of LA in our paradigm. In accordance with our previous results, the insertion of a silence during the playback of the movement-evoked sound led to a significant increase in freezing in control animals. However, inhibition of LA during the silence interval disrupted the observed increase in freezing. Moreover, there was a significant difference in the percent time spent freezing during the silence between the ArchT and Ctr for light groups, showing that activity in LA is necessary for the display of defense responses triggered by the cessation of movement-evoked sounds.

Most of what is known about auditory cues that trigger defense behaviors has been unraveled by AFC studies using mostly artificial sounds (Maren S 2004[5], Pape H 2010[6], Herry C 2014[7]). The LA has been widely implicated in the expression of defense behaviors by these studies, but knowledge about its role in processing more natural sounds is very limited (Sadananda M 2008[22], Parsana AJ 2011[21]). Interestingly, a recent study shows that innate defense behaviors can be triggered by a broadband sound through a pathway comprising the auditory cortex and the inferior culliculus (Xiong XR 2015[24]). This finding raises the possibility that other pathways independent of LA are involved in fear responses triggered by sounds. The auditory stimulus used in our experiments is quite different from most of the stimuli used in other

studies, since is the cessation and not the onset of the sound that triggers freezing. Given this difference it was possible that neuronal pathways independent of LA would underlie the defense behavior observed. However, we found that activity in LA is necessary for freezing triggered by the cessation of the sound of movement. This result shows that the LA is necessary for the display of learned acoustic defense behaviors triggered by stimulus of very different nature, ranging from pure tones to broadband modulated sounds and responds both to the onset and offset of an auditory cue.

Importantly, in our experiments we showed that activity in LA is necessary for the display of freezing. However, we cannot conclude that learning results from synaptic plasticity in LA. Prior studies have shown that long-term synaptic plasticity in the LA is required for memory consolidation of fear conditioning (Rodrigues SM[29]). The LA is then a likely place for memory formation and storage in our paradigm. However, neuronal plasticity could occur somewhere else in the fear circuit and activity in LA would just reflect a read out of those plastic changes. Further experiments addressing where plasticity takes place and the mechanisms underlying it are needed, in order to better understand the neuronal mechanisms underlying the display of defense behaviors triggered by the cessation of the sound of movement.

Although there is a vast literature concerning behavioral and neuronal changes triggered by the presentation of sounds in the context of fear (Seyfarth RM 1980[1], Weinberger NM 2004[30], Mongeau R 2003[31], Bordi F. 1993[8], Xiong XR 2015[25]) much less is known about the effects of its disappearance. However, the termination of an auditory object can convey important information about the disappearance of its source from the environment. Cells that respond to the offset of sounds have been reported along the auditory pathway and have been suggested to be important for

sound localization, auditory scene analysis and communication (Kopp-Scheinpflug C 2011[32] , Scholl B 2010[33] , Toronchuk JM 1992[34], He J 2011[25], Bordi F 2003[8]). In order to address the neuronal pathways underlying the detection of the sudden termination of the movement-evoked sound we looked at the expression of *c-fos* in different subnuclei of the auditory thalamus. We found a significant increase in the number of labeled cells in the MGd in rats exposed to the silence inserts compared to rats exposed to the continuous playback of the movement-evoked sound. This increase was particularly marked in the more dorsal part of this subnucleus. Importantly, an early study of electrophysiology in anaesthetized rats reported offset cells in several subnuclei of the auditory thalamus, with the highest percentage of these responses localized in the MGd (Bordi F 1993[8]). This is in agreement with our results, supporting the hypothesis that cells in this subnucleus have offset responses triggered by the termination of the movement-evoked sound. However, we cannot exclude that some of these cells have onset responses triggered by the resumption of the movement-evoked sound. To address this question electrophysiology studies in freely behaving animals are needed. In addition, offset cells in the auditory cortex that also send projections to the LA, may contribute to freezing triggered by the cessation of movement-evoked sound.

In the present work we found that activity in LA is necessary for the display of freezing triggered by the cessation of a movement-evoked sound. We also found an increased number of *c-fos* labeled cells in the MGd in rats displaying freezing in response to this auditory stimuli. We further confirmed that cells in this subnucleus of the thalamus project to the LA. Therefore, we hypothesize that the cessation of the movement-evoked sound triggers offset responses in cells of the MGd, that through their projections to the LA drive activity in this

nucleus. The activity in LA would then drive freezing through the activation of its downstream targets responsible for the display of learned defense behaviors. However, we cannot prove that the cells that hypothetically respond to the cessation of the sound are the ones that project to LA. To address this question we could repeat the *c-fos* experiments in animals with previous injections of CT-B in LA. A double *c-fos* /CT-B labeling would then provide further evidence that cells that respond to the auditory stimuli project to LA. However, such experiments would still not allow the distinction between on and offset cells, that can only be addressed through electrophysiology or calcium imaging techniques.

A way to test if neuronal activity in the MGd is necessary for the display of fear triggered by the cessation of the movement-evoked sound would be to optogenetically inactivate this subnucleus during the silence periods. If such inactivation disrupts freezing during silence we could conclude that neuronal activity in the MGd is necessary for freezing triggered by silence. The optical inactivation of the terminals of the MGd neurons in the LA would further show that this defense behavior rely on the direct projection from the MGd to the LA.

With the present study we've shown that activity in the LA is necessary to drive defense behaviors triggered by a cue that represents changes in the behavior of conspecifics. Given that activity in LA is necessary for the display of these responses, it is likely that there are shared neuronal pathways between social and non-social cues at least in what concerns learned defense behaviors triggered by sounds. We also found a putative subnucleus in the auditory pathway that may respond to this cue, bringing more insights into the neuronal pathways underlying transmission of fear. Finally, the present task may be useful for the study of ethological behaviors triggered by the cessation of

sounds, an area still largely unexplored.

MATERIALS AND METHODS

Subjects

Naïve male Sprague Dawley rats 300–350 g for *c-fos* experiments (Harlan) and 200-250g for optogentic experiments (Charles Rivers) were obtained from a commercial supplier. After arrival animals were pair housed in Plexiglas top filtered cages and maintained on a 12 h light/dark cycle (lights off at 8:00 p.m.) with *ad libitum* access to food and water. All behavioral procedures were performed during the light phase of the cycle. For the *c-fos* experiments, animals were kept in pairs and acclimated for at least one week before experimental manipulation and handled for 2 days before each experiment. Animals used in the optogenetic experiments were separated 4-6 days after arrival and subjected to virus injection and/or optic fiber implantation surgery. After this procedure animals were kept alone in Plexiglas boxes for 4 weeks in case of virus injections and 2 weeks in case of fiber implants before experimental manipulation. During this period animals were handled at least twice a week, one of them together with another rat.

The Champalimaud Neuroscience Programme follows the European Guidelines of animal care. The use of vertebrate animals in research in Portugal complies with the European Directive 86/609/EEC of the European Council.

Viral Vectors and Neuronal Tracer

Adeno-associated virus containing ArchT (AAV2/5 CAG-ArchT-GFP 1,3 $x10^{12}$ vg/ml) was produced by and purchased to University of North Carolina (UNC) vector core facility. The neuronal tracer Cholera Toxin subunit B Alexa Fluor 555 Conjugate (CT-B Alexa 555, 1 mg/mL) was produced by and purchased to Life Technologies.

Stereotactic surgery

Animals were anaesthetized with Isoflurane (3% for induction, 2% for maintenance Vetflurane 1000mg/g, Virbac) and placed in a stereotaxic apparatus (David Kopf Instruments). Small craniotomies were made using standard aseptic techniques. For the optogenetic experiments, animals of the ArchT group were targeted bilaterally to the LA (stereotaxic coordinates from Bregma, anterior–posterior: −3.3 mm, dorsal– ventral: -8.1, medial–lateral: 5.2 mm; Paxinos G 2007 [35]) using stainless steel guide cannulae (24 gauge; Plastics One). Following cannula guide placement, 0.3 – 0.4 μL injections of rAAV5-CAG-ArchT-GFP (diluted 1/3 in sterile PBS, final concentration 4,3 x 10^{11} vg/ml) were made through a stainless steel injection cannula (31 gauge; Plastics One), which protruded 1.0 mm beyond the tip of the guide cannula and was attached to a Hamilton syringe via polyethylene tubing. Injections were made at a rate of 0.02 μL/min, which was controlled by an automatic pump (PHD 2000; Harvard Appartus) and the injector was left in place for 10 min postinjection. After injection, cannulas were removed and optical fibers (200μm, 0.37 numerical aperture, Doric lenses) were implanted in the LA (stereotaxic coordinates from Bregma, anterior–posterior: −3.3 mm, dorsal–ventral: −8.15 or 8.05 mm, medial–lateral: 5.2 mm) and affixed to the skull using stainless steel mounting screws (Plastics One, Inc.) and dental cement (TAB 2000, Kerr). Animals were kept on a heating pad throughout the entire surgical procedure. Post-operative care included subcutaneous injection of 0,3 ml of Dolorex (Butorphanol Tartrate, 2mg/kg, Intervet) for analgesia purposes, and administration of an antibiotic (Bacitracin 500 UI/g + Vitamin A UI/g) in the skin around the implant. Rats were kept for 4 weeks before any behavioral manipulation to allow maximal expression. Animals of the Control for virus group were subjected to the same procedure. Animals of the Control for virus

were subjected to the same procedure however no virus was injected.

For retrograde tracing of auditory thalamus projection cells, CT-B Alexa 555 (0,2 μL) was bilaterally injected to LA as previously described and allowed for 6 days for sufficient retrograde transport.

All injection sites and fiber placements were verified histologically and rats were excluded if either were mistargeted.

Histological processing

Animals were deeply anhestetized with pentobarbital (600 mg/kg, i.p.) and transcardially perfused with PBS (0.01M), followed by ice-cold 4% paraformaldehyde (Paraformaldehyde Granular; cat#19210; Electron Microscopy Sciences) in 0.1 M phosphate buffer (PB) (PFA-PB). Brains were removed and postfixed in 4% PFA-PB and kept at 4ºC. Coronal sections of 40 μm containing the LA (3.20 to 3.90 posterior to bregma) and/or the auditory thalamus (-5.60 to -6.60 posterior to bregma, Paxinos *et al.* 2007[35]) were cut, and half of the intercalated slices were mounted using moviol. The other half was collected in PBS and stored in antifreeze solution at -20ºC.

For cannula placement verification (control animals in the optogenetic experiments) and visualization of the subnucleus of the thalamus (CT-B injections in LA), selected slices were labeled with an anti NeuN antibody (rabbit; ab177487; abcam). Brain section were first washed in PBS (3 x 10 min) and PBST (0,04% Tween 20 in PBS; 1 x 10 min), and blocked in 10% normal goat serum (Milipore) in PBS-T for 2h, room temperature. Next, slices were incubated with the 1ry antibody anti-NeuN (1:2000) in PBS-T with 2% NGS at 4ºC overnight. In the next morning, sections were washed in PBS-T with 2% NGS (3 x 10 min) and incubated with the 2ry antibody Alexa Fluor 488 (1:1000; goat; ab150089; abcam) in PBS-T with 2% NGS for 2h room

temperature. After washing with PBS (3 x 10min), slices were mounted onto glass slides (Superfrost Plus, Thermo Scientific) with Moviol. Images were taken in the Widefield Fluorescence Sacnning Microscope (Zeiss Axioimager M2) and in the Confocal Laser Point-Scanning Microscope (Zeiss LSM 710).

Immunohistochemical experiments examining *c-fos* expression

2 h after the beginning of the behavioral experiments animals were deeply anaesthetized with pentobarbital (600 mg/kg, i.p.) and transcardially perfused with PBS, followed by ice-cold 4% PFA-PB. Brains were removed and postfixed in 4% PFA-PB for 24 h and subsequently cryoprotected in 20% glycerol (J.T.Baker) in 0.1 M PB for 72 h at 4ºC. Using a sliding microtome, sections of 40 μm containing the auditory thalamus (5.40 to 6.40 posterior to bregma) were cut and collected in PBS. Next, sections were transferred to a 0.1% sodio azide (Sigma) in PBS solution for storage. The immunohistochemical staining was performed simultaneously for all brain sections analyzed. Staining was performed in free-floating sections, processing every sixth section. Sections were washed 3x10 min with PBS, incubated for 10 min with 0,9% H_2O_2 (Sigma-Aldrich), washed again 3x10 min in PBS and blocked in PBS with1% bovine serum albumin (BSA) (cat#A7906, Sigma-Aldrich) and 0.1% Triton X-100 for 1h room temperature. Slices were then incubated overnight at room temperature with anti *c-fos* 1ry antibody (1:500; rabbit; sc-52 Santa Cruz Biotechnology) in PBS with1% BSA and 0.1% Triton X-100. The next morning, sections were washed 3x10 min with PBS and incubated with goat anti-rabbit byotinilated 2ry antibody (1:1000; Cat#4050-08, Southern Biotec) in PBS with 1% BSA and 0.1% Triton X-100 for 1h room temperature. Sections were washed 3x10 min in PBS, incubated with Horseradish Peroxidase Streptavidin (1:300; cat#SA-5004; Vector Laboratories) in PBS with 0.2% Triton X-100 for

1h room temperature, washed 3x10 min in PBS-B and developed in diaminobenzidine tablets (DAB) (cat# D4418-50 SET; Sigma) for 3 min. Sections were mounted on electrostatic slides, air dried, dehydrated in ethanol and xylenes and coverslipped with DPX.

Brightfield images were taken in Zeiss Axioimager M1 microscope equipped with a CCD camera (Hamamatsu C8484), with objective 20x/0.80. Sections from comparable anteroposterior levels were selected for scoring and cell counts were scored using NIH Image J software. For analysis, cell counts for each region were averaged into a single score for each rat.

For the analysis along the AP axis of the MGd we divided the sections in anterior (sections including and posterior to 5.64 until 5.76 (including) relative to bregma), medial (sections posterior to 5.76 until 6.00 (including) posterior to bregma) and posterior (sections posterior to 6.00 until 6.24 (including) relative to bregma) and averaged the *c-fos* labeled cells in those slices.

Behavioral Apparatus

Two distinct environments were used in this study, the conditioning chamber and the test box, which were located in different procedure rooms. The conditioning chamber (model H10-11R-TC, Coulbourn Instruments) had a shock floor of metal bars (model H10-11R-TC-SF, Coulbourn Instruments) and was inside a high sound isolation chamber (Action automation and controls, Inc). In this chamber a precision programmable shocker (model H13-16, Coulbourn Instruments) delivered the foot-shocks. The sound was calibrated using a Brüel and Kjaer microphone (type 4189) and sound analyzer (hand held analyzer type 2250). The test box consisted of a two partition chamber made of clear Plexiglas walls (60cm wide x 34cm height x 27cm depth)

(Gravoplot). The chamber was divided in two equal halves by a clear Plexiglas wall, but only one side of the box was used. The floor of the chamber contained a tray with bedding. The test box was placed inside a sound attenuation chamber, (90cm wide x 45cm height x 52.5 cm depth) made of MDF lined with high-density sound attenuating foam (MGO Borrachas Técnicas) and a layer of rubber. Inside the sound attenuating box it was placed a set of 2 speakers placed next to each other (HP multimedia 2.0 speakers) used to playback the previously recorded movement-evoked sounds. This sound resulted from the movement of a naïve rat inside the test box with a tray with bedding. This sound was posteriorly filtered to remove affiliative calls emitted by the rat. The two chambers differed also in the illumination conditions, with illumination on the conditioning chamber and no light on the test box. In addition, the boxes were cleaned with two cleaning fluids with distinct odors. The rats' behavior was tracked by a video camera mounted on the ceiling of the attenuating cubicle in the case of the conditioning chamber, and by a CCTV camera (Henelec 300, Henelec) mounted on the back wall of the attenuation box in the case of the test box. A surveillance video system (Color Quad System, Henelec) connected to a video acquisition system (Dazzle Dvd Recorder HD) was used to record and store all videos on hard disk and freezing behavior was automatically scored using FreezeScan from Clever Sys.

For the optogenetic experiments, the light delivered by a 500mW 556nm laser (Changchun New Industries Optoelectronics Tech. Co., Ltd) was controlled by a mechanical shutter (SH05, Thorlabs) connected to a TTL pulse generator (SC 10, Thorlabs)

Behavioral procedures

All rats were exposed to one of the two environments for fifteen minutes on the first four days of experiment (two exposures for each environment in total, in alternating days). Movement-evoked sounds were played during exposure to the testing environment.

Optogenetic experiments

After the exposure days, experienced rats were placed in the conditioning chamber and received three shock presentations (1mA, 0.5s) with an average intertrial interval of 180s. After the training session animals were returned to their home cage. The next day, a fiber optic cable terminating in 2 ferrules (Branching Fiber-optic 200μm, 0.22 numerical aperture, Doric lenses) was connected to the chronically implanted optic fibers. The animals were then placed in the test box and the recorded sound of movement was played throughout the test session (five minutes), except during the one-minute period of silence. Laser illumination (estimated 30mW at the tip of the fiber) started 10 sec before the silence and lasted until 5 sec after the resume of the playback of the movement-evoked sound. After the test session, animals returned to their homecages. If due to generalization to the two environments animals were freezing before the silence inserts, animals stayed in the test box for 5 min of continuous playback, and tested the next day.

c-*fos* experiments

Experiments were performed as described above, however in the Silence group two periods of silence with the duration of one minute each were inserted during the playback of the movement-evoked sound with a period of movement-evoked sound playback of 3 minutes in between (total duration of

the session 9 min). For the control group (Continuous Playback) the recorded sound of movement was played throughout the entire test session.

Results are presented as the average number of c-fos labeled cells per 10^5 μm^2.

Statistical Analysis

We used a Shapiro-Wilk test to access the normality of our data.

The variables in the optogenetic and *c-fos* experiments were not normally distributed and sample sizes are small, so we used non-parametric tests only.

In the optogenetic experiments we focused on the silence gaps that lasted for one minute, and used as baseline the minute immediately preceding the silence interval. In this manner we ensure that both measures have the same sampling time. For comparisons between groups during baseline and silence we used a Mann-Whitney test and corrected for multiple comparisons. For comparisons within groups we conducted a Wilcoxon signed-ranked test.

For the *c-fos* behavioral data we averaged the percentage of freezing during the two periods of one minute preceding the silence (Pre Silence) and the two periods of silence (Silence). For comparisons within groups we conducted a Wilcoxon signed-ranked test. For comparisons between groups of the *c-fos* labeled cells we used a Mann-Whitney test. To analyze the effect of position along the AP axis and exposure to silence in the expression of *c-fos* we conducted a Mix-model ANOVA given that our data was normally distributed. For post-hoc multiple comparisons we used a Fisher (LSD) test.

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CHAPTER 4 - GENERAL DISCUSSION

Sensory Processing in Social Transmission of Fear

Social Transmission of Fear (STF) can be seen as a phenomenon where individuals in a group react to the display of defense responses by their conspecifics. Understanding the neuronal mechanisms underlying this phenomenon is not only a way to address how social information is processed in the brain, but also when studied in larger groups of animals investigate group dynamics in response to external threats. However, an essential point for the better understanding of this question is how information is exchanged between individuals.

A way to start addressing this question is to look at the sensory cues used by animals to detect fear in others. Field and laboratory studies have shown that a variety of sensory cues can be used, from olfactory to visual or auditory (Ono M 2003[1], Hingee M 2009[2], Mirza RS 2003[3], Wilson DR 2004[4], Seyfarth 1980[5], Mineka S1993[6], Olsson A 2007[7]). Interestingly, it has been proposed that at least in the context of social learning about threats there is preferential learning about some stimuli given the class of the animals namely visual and olfactory cues seem to be preferentially used by fish, while eutherians mainly rely in visual and acoustic alarm cues (Griffin A S 2004[8]).

Laboratory studies using rodents as an experimental model have implicated both visual (Jeon D 2010[9]) and auditory cues, namely alarm calls (Kim E 2010[10] but see Atsak P 2011[11]) in STF. In our study we probed for both these cues, and we did not find any evidence that they are necessary for observer rats to respond to the fear behavior of the demonstrators. These differences may be due to the species used in the study as well as to the behavioral paradigm. Jeon D *et al* (Jeon D 2010[9]) used mice as experimental model, and observers were directly witnessing the exposure of the

demonstrators to aversive footshocks while separated by a transparent partition. When replacing for an opaque partition, the authors report a significant decrease of freezing by the observers, suggesting that direct visual observation is important for transmission of fear and social learning about context. However, residual freezing was still reported, most likely due to other cues such as olfactory or auditory. An important aspect of this study is that although the authors say that these later cues can still be exchanged under the shock floor, they are most likely attenuated right in the first experiment by the transparent partition. Also, the fact that observers are witnessing a conspecific being shocked can make the visual information more striking since the delivery of the unconditional stimulus (US) makes animals jump and run around the conditioning box. Therefore, it is possible that the experimental design increases the strength of visual information making it the most reliable cue.

Kim E *et al.* (Kim E 2010 [10]) proposed that 22Khz Ultra Sonic Vocalizations (USVs) are a crucial feature of STF given the positive correlation between the onset of USVs emitted by observers during their exposure to footshocks and observational freezing. Permanent lesions or temporary inactivation of the auditory thalamus in observers during exposure to footshocks, disrupted observational freezing suggesting that auditory cues are necessary for this behavior. However, in our study we found that alarm calls were not necessary for STF. While in Kim's study demonstrators strongly vocalized during the social interaction when presented with the conditioned stimulus (CS), in our study this only happened in 1 out of 8 pairs. Hence, in 7 pairs the observers froze while witnessing the freezing of demonstrators in the absence of alarm calls. In another work investigating the role of alarm calls, Atsak P. *et al* (Atsak P 2011[11]) did not found a significant correlation between USVs emitted by

demonstrators and the percentage of freezing of experienced rats directly witnessing the demonstrators being shocked. In addition, the authors found that alarm calls induce similarly low levels of freezing in naïve and experienced rats (and also indistinguishable from a control sound) (but see Parsana AJ 2012[12]). Interestingly, when the authors played back the unfiltered recordings of the STF test (USVs together with other audible sounds) they found that experienced rats froze significantly more to this auditory cue than naïve animals. So it is possible that in Kim E *et al* (Kim J 2010[10]), the inhibition of the auditory thalamus during exposure to shock affects the processing of both USV's and other auditory cues that may be necessary for STF. That would be in accordance with our results, since we found an auditory driven defense behavior that depends on a prior aversive experience with footshocks, but where alarm calls do not play a role. Most likely rats will use both auditory cues to access the defense behaviors of conspecifics.

It has been previously shown that both mice and rats release alarm pheromones that trigger both physiological and behavioral changes in naïve individuals (Kiyokawa Y 2006[13], Brechbuhl J 2009[14]). These behavioral changes include the display of defense behaviors like freezing, decrease of exploratory activity and increased risk assessment. The importance of olfactory cues in SFT has also been reported in a recent work showing that aversive learning in pups towards a novel olfactory cue (CS) can be achieved by pairing the CS with the odor of a frightened mother (Debiec J 2013 [15]). However, in the above-mentioned studies looking at STF in pairs of adult rats they do not seem to be sufficient (Kim E 2010[10], Pereira AG 2012[16]) or necessary (Pereira AG 2012[16]) and may only have residual effects (Jeon D 2010[9]). One hypothesis that may explain this is the temporal relationship between the cue and the

behavior of the animal. After olfactory cues are released they can stay in the environment for long periods of time and provide very little information about the strategy adopted by their releaser. In contrast, visual and auditory cues resulting from movement (or absence thereof) are directly coupled to the defense behaviors of the animal and can be used to access the strategy adopted by the other individuals of the group (ex. stay immobile or flee, or transitions between these states).

Although these different studies point to the importance of different sensory information, in natural conditions animals are likely to integrate the multiple cues provided by others as well as their own private information. Olfactory cues may trigger a state of alertness while auditory and visual cues may provide more detailed information about the best behavioral output to adopt (e.g. different alarm calls given the type of predators in vervet monkeys and ground squirrels (Seyfarth RM 1980[5], Mateo JM 1996[17])). However, some cues may become more relevant than others given the situation (e.g. auditory cues if individuals are not in close proximity) or their reliability.

Sound of Movement

In our study we found that the cessation of the movement-evoked sound is both necessary and sufficient to trigger defense behaviors in experienced rats. We have shown it in two different experiments: during the social interaction when the playback of the movement-evoked sound disrupted freezing in observer rats, and in the playback of movement-evoked sound experiment where a rat alone displayed freezing during the silence intervals even in the absence of other sensory cues. One possible explanation could be that during prior experience with footshocks animals become conditioned to the silence,

since they receive footshocks while in silence given their own immobility. However, in a control experiment for the playback of movement-evoked sound rats froze after the cessation of the movement-evoked sound even when a continuous 2KHz pip was played. This result leads to the hypothesis that the cessation of the movement-evoked sound, and not silence per se, triggers freezing. Also, in a control experiment for the social interaction, the playback of a continuous 2KHz pips that disrupted the silence caused by the immobility of both demonstrators and observers did not decrease the freezing of the observers. In fact, there was a slight but significant increase in freezing of both rats. This could be due to some generalization of the demonstrators to the 2KHz pips given that they were previously conditioned to a 5KHz sound. The increased freezing of demonstrators could have influenced the freezing of observers, making the interpretation of this experiment difficult. However, it is still an indication that not any change in the auditory scene decreases freezing and that most likely it is related to the information provided by the movement-evoked sound.

This sound results from the movement of a naïve rat in a tray with bedding; the same that rats normally have at their own cages. It is most likely that during their lifetime in the home cage animals associate their own movement to this sound, through sensory motor integration. Also, there are other rats in their surroundings producing the same rustling sounds, so that the movement-evoked sound is part of the everyday acoustic environment of these rats.

It is then possible that this sound becomes associated with safety. According to Rogan M *et al* (Rogan M 2005[18]) "learned safety is a special case of conditioned inhibition characterized by the reduction of conditioned fear

responses by a signal that has been negatively correlated with aversive events". The authors report that when mice are exposed to an auditory CS explicitly unpaired to the footshock, the presentation of the CS (safety signal) decreases freezing responses to the context (in this case the predictor of the US). This data can be interpreted as a reduction of conditioned fear induced by a safety signal. A similar mechanism could underlie our experiments, where the playback of the movement-evoked sound during the STF experiments reduces the conditioned fear in observers triggered by the freezing of the demonstrators. Rogan M *et al* (Rogan M 2005[18]) also reports that safety conditioning leads to a decrease of the slope and amplitude of CS- evoked field potentials in the Lateral Amygdala (LA) which may reduce the excitation of efferent structures responsible for driving freezing. In our experiments we found that activity in LA is necessary for the display of defense behaviors triggered by the cessation of the movement-evoked sound. It is then possible that activity in LA triggers freezing but the playback of the movement-evoked sound affects this activity influencing downstream structures responsible for the display of this specific defense behavior.

It is however important to make a distinction between the classical safety signals and the movement-evoked sound used in our experiments. A safety signal is "a specific class of conditioned inhibitor; as a result of Pavlovian conditioning, it prevents or reduces the expression of fearful behaviors normally observed in the presence of an excitatory CS that had been paired with aversive unconditioned stimuli US. Thus, the first requirement for a safety signal is that it must come to inhibit the conditioned fear response as a result of learning (Christianson JP 2012[19]). In most paradigms used to study safety signals, animals are trained in tasks where there is a clear unpairing between the signal and the aversive outcome, or the signal occurs after the aversive

event is over. In the case of the movement-evoked sound, no such Pavlovian process (where the presentation of the stimulus signals the absence of an aversive event) occurred. In the case of movement-evoked sound, the acoustic cue becomes a safety signal due to the experience of the animal throughout its life.

The role of prior experience

In the present thesis we found that rats that had a prior aversive experience with footshocks, but not naïve animals, freeze in the presence of demonstrator rats displaying fear responses. This result is in accordance with other studies showing that prior experience with footshocks is necessary for STF (Kim E 2010[10], Atsak P 2011[11], Church MR 1959 [20], Sanders J 2013[21]). However, the work of Jeon D *et al.* using B6 mice reports that naïve observers significantly increased their levels of freezing while witnessing demonstrators being fear conditioned (Jeon D 2010[9]). This could be due to species differences, but a more recent work by Sanders J *et al.* (Sanders J 2013[22]) using the same experimental model also found that experienced, but not naïve mice, significantly increased their levels of freezing while witnessing a conspecific being footshocked. Importantly, the frequency, duration and intensity of footshocks in Jeon D *et al.* (Jeon D. 2010[9]) was higher than in Sanders *et al.* (Sanders J 2013[22]), suggesting that the intensity of the fear responses witnessed by the observer may influence its behavior. It should also be noted that in our behavioral paradigm, as well as in Kim EJ *et al.* (Kim EJ 2010[10]), observers do not directly witness demonstrators being footshocked. Transmission of fear occurs while the demonstrator is exposed to the CS to which it was previously conditioned, that mainly triggers freezing and emission of alarm calls in the absence of pain responses (but see Atsak P 2011[11]).

Thus, although we found that STF only occurs in experienced rats, we cannot exclude the possibility that the nature of the aversive responses displayed by the demonstrator and its intensity may be an important factor in observational fear. In fact, it has been shown that the intensity of the demonstrators' fear responses is positively correlated with the responses of the observer (for review see Crane LA 2013[22]). In the framework of social learning about threats it is thought that the fear displayed by an individual in response to a fear eliciting stimulus can serve as an US to the observers (Mineka S 1993[6]). Higher levels of fear can act as stronger US, triggering defense behaviors even in naïve individuals.

Studies done under laboratory settings found that social learning about threats can occur between fear conditioned partners and naïve animals (Jeon D 2010[9], Jones CE 2014[23], Bruchey AK 2010[24], Chen Q 2009[25]). These tests addressed how genetic background, time of co-housing or social interaction during the fear conditioning by proxy task (during which the CS to which the demonstrator was fear conditioned is played back in the presence of the observers) influence social learning about threats. It would be interesting to further address the role of prior experience, investigating if prior exposure to a similar aversive event would influence learning.

Given that in our experimental paradigm we found that rats with a prior aversive experience with footshocks, but not naïve animals, freeze in the presence of fearful demonstrators, we are in the presence of a learned defense behavior. Most of what is known about auditory cues that trigger defense behaviors has been unraveled by Auditory Fear Conditioning (AFC) studies using mostly artificial sounds (for review see Herry C 2014[26], Pape H 2010 [27]).

In these studies, subjects learn to associate a neutral sound (CS) with an innately aversive stimulus (US). The later presentation of the CS alone triggers defense behaviors. The auditory stimulus used in our experiments is quite different from most of the stimuli used in these studies, since it is the cessation and not the presentation of the sound that triggers freezing. This poses the question of how prior experience with shocks leads to the display of defense behaviors triggered by the cessation of the movement-evoked sound. During prior experience, the rat is moving around the box when suddenly it receives a footshock triggering an initial burst of activity characterized by jumping and running. Afterwards, rats tend to freeze for long periods, being the freezing reinforced by the delivery of the following shocks. The US is then associated with the environment, but possibly also with the silence in the box or with immobility. In the testing day, the animal responds to an auditory signal that suggests the transition from movement to immobility, being now immobility and/or silence associated with an aversive state. Brown T *et al.* (Parsana AJ 2012[12]) reported that rats freeze to the presentation of USVs alarm calls when they had prior aversive experience with shocks. Given that rats emit alarm cries while being shocked, the authors proposed a mechanism of "autoconditioning", where rats hear their own USVs and associate them with a concomitant state of fear due to the delivery of the US. Afterwards, rats generalize to 22 Khz USVs produced by other individuals. This mechanism is in agreement with a study investigating the role of USVs in STF (Kim J 2010[10]). Although in our case USVs do not play an important role in STF (Pereira AG 2012[16]), it is possible that a mechanism similar to autoconditioning to alarm calls may happen. One possibility is that the delivery of the footshocks makes the animal jump and run, being this burst of activity followed by freezing. Cells in the auditory pathway may then respond to the cessation of the sound of

movement evoked by jumping and running, and synapses from these cells may get potentiated to downstream targets like LA. When afterwards (either during the STF or the playback of movement-evoked sound test) silence is instated in the environment, cells that responded to the cessation of sounds during prior experience may generalize their response to the cessation of other movement-evoked sounds. Their previously reinforced connection to downstream targets may lead to freezing.

Neuronal Pathways in Social Transmission of Fear

In the present work we found that activity in the LA is necessary for the display of freezing as a measure of fear in response to the cessation of the movement-evoked sound. Given that this cue is necessary for STF in our paradigm, the LA is likely involved in fear responses driven by this social acoustic stimulus. Previous studies have also shown that inhibition of LA in observers affects both transmission (Jeon D 2010[9]) and socially acquired fear (Jeon D 2010[9], Debiec C 2014[15]). In particular, the study by Jeong D. *et al* (Jeon D 2010[9]) has shown that the inactivation of the LA abolished freezing in a mouse observing another mouse receiving footshocks. Moreover, the authors report that a significant synchronization of theta rhythm between the Anterior Cingulate Cortex and the LA are necessary for the acquisition of fear through observational learning. Together, these studies provide evidence that activity in LA is important for the display of freezing whether the animal directly encounters the threat (Martinez RC 2011[28], for reviews see Herry C 2014[26], Pape H 2010[27]) or uses information provided by others about impending danger.

Activity in LA has been shown to be important for both learned and innate defense behaviors triggered by auditory and visual cues (Wei P 2014[29], for

reviews see Herry C 2014[26], Pape H 2010[27], Gross C 2012[30]). For acoustic driven defense behaviors, most of what is known results from studies of AFC where animals learn that a given acoustic cue predicts danger. These studies have demonstrated that the LA is necessary for learning, storage and expression of defense behaviors triggered by sounds (reviewed in Herry C 2014[26], Pape H 2010[27]). However, most of these studies used artificial sounds like pure tones or white noise. In our experiments, we found that the cessation of an ecologically relevant sound that results from the movement of conspecifics is sufficient to trigger freezing in experienced rats. The nature of our stimulus, that encompasses a sudden transition from a modulated broadband sound to silence, is very different from most auditory stimuli previously used. Taking advantage of optogenetic techniques, that allow neuronal manipulations with temporal precision, we inactivated LA during the periods of silence. We found that inactivation of this subnucleus specifically during this period disrupts freezing triggered by the cessation of the movement-evoked sound. This result suggests that LA is important in learned acoustic defense behaviors whether they are triggered by the presentation or cessation of a sound. Interestingly, a recent paper reported that in mice flight could be induced by a broadband loud sound through activation of corticofugal neurons in the Auditory Cortex. Further characterization of the neuronal pathways underlying this defense behavior show that these neurons drive neuronal activity in the cortex of the Inferior Culliculus (ICx) that through its connections to the midbrain defense system mediate the escape behavior (Xiong XR 2015[31]). This pathway is then sufficient to induce flight and is independent of the amygdaloide nucleus. This result, together with what is already known about LA, suggests that distinct pathways may exist for fear driven by an innate or a previously learned acoustic cue.

Synaptic potentiation of both thalamic and cortical auditory inputs to LA has been previously shown, elucidating the mechanism underlying AFC. At the thalamic level, the role of the medial portion of the Medial Geniculate Body (MGm) and posterior intralaminary nucleus (PIN) has been further investigated, and several studies suggest that plasticity between the auditory thalamus and the LA is important for learned auditory-triggered defense behaviors (for review see Maren S 2004 [32], Ehrlich I 2009 [33], Herry C 2014[26], Pape H 2010[27]). Besides the MGm and the PIN, other subnuclei of the auditory thalamus like the dorsal division of the Medial Geniculate Body (MGd) and the suprageniculate nucleus (SG) have direct projections to the LA (Doron NN 1999[34], Namura S 1997[35]). Importantly, previous studies found offset responses in several of these nuclei (Bordi F. 1993[36], He J 2001[37]). In our experiments we found that an acoustic stimulus that signals the cessation of movement-evoked sounds triggers freezing in rats, and the expression of this defense behavior depends on LA. Given the previous characterization of the auditory thalamus, we hypothesized that offset cells in this structure may detect the cessation of the movement-evoked sound and through the direct inputs to LA drive activity in this afferent structure triggering freezing.

In order to unravel the neuronal pathways underlying the detection of the cessation of the movement-evoked sound, we performed a *c-fos* experiment looking at differences in neuronal activity in experienced rats exposed to the movement-evoked sound with or without silence inserts. We found a significant increase in *c-fos* labeled cells in the MGd of rats exposed to the silence inserts. This is in accordance with a previous electrophysiology study performed in anaesthetized rats that reported the highest percentage of cells with offset activity in the MGd (Bordi F 1993[36]). However, it should be noted that cells in this region (as well as in the other subnuclei) also respond to the initiation of

sounds, so it is possible that activity in some of these cells is triggered by the resumption of the movement-evoked sound. In any case, this result gives indications that activity in this region might be modulated by transitions in the auditory scene, and is a candidate area to signal the cessation of the movement-evoked sounds.

Finally, given that we are in the presence of a learned defense behavior, we further hypothesized that synaptic changes occur between the MGd and the LA during prior experience. These changes will lead to a potentiation of the synapses between cells that respond to the cessation of sounds and downstream pyramidal neurons in LA. This potentiation would underlie the defense behaviors triggered by the posterior presentation of our acoustic stimuli.

Future directions

The cessation of the movement-evoked sound triggers defense behaviors in experience rats, being the display dependent on LA activity. We suggested that this sound can act as a safety cue and its disappearance as a signal of danger. The behavioral output that we obtained in the STF experiment where the playback of the movement-evoked sound disrupts fear in observers, could then be explained by changes in neuronal activity in LA. Rogan M *et al.* (Rogan M 2005[18]) reports that safety conditioning leads to a decrease of the slope and amplitude of CS- evoked field potentials in LA. In our experiments, freezing of the demonstrator leads to the cessation of the sound of movement, and this cessation may trigger neuronal activity in the observers' LA, thereby driving freezing. The subsequent playback of the movement-evoked sound could then lead to the decrease of neuronal activity in LA, disrupting the defense behavior. To address if such changes in neuronal activity occur in LA, and how

they are correlated with the behavior of the observer rats, one could perform electrophysiology recordings in LA during this paradigm. This experiment would give us further insight about the nature of the movement-evoked and how it is processed in the brain. We cannot exclude the hypothesis that only its cessation modulates activity in LA. The effects of its playback (or its generation by activity of other rats) can modulate activity in other areas in the brain. In fact, Rogan M *et al.* (Rogan M 2005[18]) reports that safety conditioning concomitantly increases the slope and magnitude of CS-evoked field potentials in the Caudate Putamen, a region involved in motivational processes including reward and positive affect processing. Addressing how a single stimulus can both trigger and inhibit fear responses can give important insights about how safety and danger is encoded in the brain. Also, it is likely that its positive valence was acquired through the lifetime of the rats, which may be closer to how safety signals are acquired in natural situations.

Another important question that resulted from our study is whether activity in LA is necessary for learning during prior experience. In our experiments we showed that activity in LA is necessary for the display of freezing triggered by the cessation of the movement-evoked sound. However, we cannot conclude that learning results from synaptic plasticity in LA. In fact, plastic changes could occur somewhere else in the brain and activity in LA during expression of the fear behavior could just reflect a readout of those changes. To address this question, in further experiments we could inactivate LA during prior experience, namely from shock delivery till the initiation of freezing. Another alternative would be to block synaptic plasticity in LA by delivering to this subnucleus a transgene that encodes for the carboxyl cytoplasmic tail of GluR-1 receptors. This protein prevents synaptic incorporation of endogenous GluR1-receptors,

and it has been previously shown to impair fear acquisition in an AFC task (Rumpel S 2005[38]).

Our results together with previous studies (Doron NN 1999[34], Bordi F 1993[36]) suggest that cells in the MGd respond to the cessation of the movement-evoked sound and possibly through the direct connections with LA drive freezing. However, in order to directly address the role of MGd, further experiments are needed. Optogenetic inactivation of the MGd starting in the transition from the movement-evoked sound to silence, and lasting through this period, would provide evidence that neuronal activity in this nucleus is necessary for the display of defense behaviors. If such inactivation disrupts freezing, it would be an indication that this nucleus of the auditory thalamus is involved in the processing of the auditory cues that signals danger. To prove that information about the cessation of the movement-evoked sound is sent to LA through the direct input from MGd, further experiments where the terminals from this subnucleus to the LA are inactivated during the same period should be performed. If the inactivation of this terminals is sufficient to abolish freezing, it would indicate that information directly conveyed from the auditory thalamus to LA is necessary for freezing triggered by the cessation of the sound of movement. Finally, to prove sufficiency, a strategy using transynaptic retrograde tracers driven by neuronal activity would be of major interest. A way to address if the inputs from one afferent region to a specific downstream structure is sufficient to drive or inhibit a given behavior, is to deliver a viral vector to the downstream structure that allows the expression of a Cre recombinase fused to a transcellular retrograde tracer protein, e.g., wheat germ agglutinin (WGA). On the input side, a Cre-dependent virus conditionally expressing the microbial opsin gene of choice (ArchT or ChR2) should also be delivered. This strategy has been used by Gradinaru V. *et al* (Gradinaru V

2010[39]), where the dentate gyrus in one hemisphere was injected with an AAV2 virus carrying a transgene that drives the expression of mCherry and the fused protein WGA-Cre. To the contralateral dentate gyrus a transgene that drives a cre dependent expression of the fused protein ChR2/YFP was delivered. The cells expressing WGA-Cre were labeled by mCherry and the retrograde transport of the fusion protein lead to the expression of ChR2 in the cells in the contralateral dentate gyrus directly connected to the cell bodies expressing mCherry. Further optogentic manipulation of the ChR2 expressing cells in the contralateral dentate gyrus directly influenced activity in the mCherry labeled cells. However, it should be noted that although this approach is quite specific it will also influence activity in other efferents of the ChR2 expressing neurons. A modification of this strategy using an activity dependent promoter like *c-fos* or *arc* (activity-regulated cytoskeleton-associated protein) would improve the specificity of this approach. Namely, in our case, the delivery of a transgene in LA that drives the expression of the fusion protein WGA-cre under the control of an *arc* promoter. At the same time, a transgene expressing ChR2 in a cre dependent way would be delivered to the MGd. During the playback of the movement evoked sound experiment, active cells in LA would express WGA-cre that would be uptaken by the terminals of the MGd allowing the expression of Chr2 only in cells directly connected with LA active cells. If synaptic plastic changes occurs between these two structures, light activation of cells in MGd should be sufficient to drive freezing. Combined with the experiments proposed above this strategy would prove that connections between the MGd and LA are both necessary and sufficient for the display of defense behaviors triggered by the cessation of the movement-evoked sound.

In fact, we tried to develop such strategy during the course of the present PhD. However, when testing the transgene used by Gradinaru V. *et al.*

(Gradinaru V. 2010[39]), we found virtually no labeling of cells in the auditory thalamus. This could be due to a deficient transport of the retrograde tracer WGA fused with cre. Further improvements of this technique could be done as follow up experiments, in order to implement the previously suggested experiments.

Overview of Empirical Findings and their Implications

The main topic of research of the present thesis is social transmission of fear, a phenomenon reported in many different species. We used rats as an experimental model system and focused on the sensory cues mediating it, as well as the neuronal pathways underlying the processing of such cues.

After successfully establishing a paradigm, we confirmed previous findings that social transmission of fear depends on the prior aversive experience with footshocks of the subjects witnessing the display of defense behaviors. This result has important implications about how individuals use information conveyed by others, suggesting that the life history of an individual deeply influences the way social information is processed. Studies performed in humans show that the presentation of unpleasant pictures triggers freezing in female subjects. Importantly, subjects who previously experienced a traumatic life event showed a more marked decrease in heart rate and increased freezing to the presentation of unpleasant pictures when compared with those who didn't experienced such event (Hagenaars MA 2011[40]). Another study also reported that the reaction to the pain of others is influenced by prior experience with the same painful stimuli (Preis MA 2012[41]). Behavioral paradigms like ours (also see Atsak P 2011[11], Kim E 2010[10], Sandres J 2013[22], Church RM 1959[20]) that use rodents as experimental models, can therefore be used to study the neuronal changes induced by prior aversive experiences and through which mechanisms they influence the subsequent response to fear or pain in others.

When looking at the sensory cues underlying transmission of fear, we found that rats do not rely on contact, visual cues or alarm calls emitted by others. Instead, they use an auditory cue that signals acoustic changes in the

environment that results from the transition from movement to immobility. We found that the playback of a sound generated by the movement of a rat is able to disrupt freezing of observers in the presence of an immobile demonstrator. However, such disturbance does not occur with the playback of a sequence of pure tone pips. Moreover, the interruption of this movement-evoked sound (by the insertion of periods of silence) is sufficient to trigger defense behaviors in rats with a prior aversive experience with shocks. These results bring interesting perspectives about how the movement and the auditory cues generated by it may be important in communication between individuals. In fact, such cues are highly reliable since they are a direct byproduct of the behavior of the animal, and consequently hard to fake. Moreover, auditory cues have the advantage of spreading fast and travel far away from the source, and therefore might be used by individuals that are not in the direct surroundings of the animals producing them. There is growing evidence showing that cues provided by movement are important for group behavior and that the noise resulting from an animals' locomotion may have a role in animal communication. In fish, it has been shown that the Lateral Line has an important function in fish schooling. The Lateral Line is a superficial sensory system that detects water displacements and its peripheral sensory cells are similar to the hair cells in the inner ear in mamals (Larrson M 2009[42]). Faucher *et al.* (Faucher 2010[43]) inactivated the whole lateral system of firehead tetras, *Hemigrammus bleheri*, and found that fish with such treatment were unable to maintain the school. The vibrations caused by body movements are also used by various mammalian species in communication, like foot-stamping in kangaroo rats, *Dipodomys*, or elephant shrews, *Elephantus rufescens* (Randall JA 2001[44]). A role for the sound of movement in intraspecies and interspecies communication has also been found in birds. Namely, wing beats with certain

characteristics might serve as predator alarm in both the mourning dove and the crested pigeon (Hingee M 2009[2], Coleman SW 2008[45]). In particular, Hingee M *et al.* (Hingee M 2009[2]) found that the presentation of a threat to crested pigeons triggers an alarm take off flight whose whistles are louder and with a faster tempo than the ones resulting from a normal take off. When playing back the sound of alarm and normal take offs to conspecifics, it was found that they used these acoustic differences adaptively since they take off in alarm only after alarmed whistles. Coleman SW *et al.* (Coleman SW 2008[45]) suggested that wing whistles may contain important information, an idea supported by Larsson M *et al.* (Larsson M 2011[46]). However, this last author proposes that the non-alarm whistles may also play a role - "the alarm whistle cannot be considered incidental. Although the non-alarm whistle may fulfill this criterion, it does produce a signal, roughly saying "no danger, just leaving". Thus, the line between incidental sounds produced as a by-product of locomotion and intentionally modulated communicative sound may not be clear". A similar effect may occur with the movement-evoked sound and its cessation. The movement-evoked sound can signal a safety state, since it is normally produced while the animal is exploring an environment. Although its production probably doesn't serve a particular function, can still provide information to other individuals in the environment. Freezing, on the other hand, has been described as a defense response quite pervasive in the animal kingdom. It has been suggested to have several advantages like optimizing perceptual and attention processes, preparing for rapid escape or defensive fighting and avoiding detection by predators (Hageenars MA 2014[47]). In a study performed in juvenile snakes, yellow-bellied racers (*Coluber constrictor mormon)*, the authors found that they attack a live cricket (*Acheta domesticus*) faster than a dead one. The authors suggest that this difference it is due to the

fact that a dead animal is harder to detect since in such cases snakes can only rely on olfactory cues. In contrast, a living animal can be detected by both olfactory and auditory cues that result from movement. Interestingly, if a live cricket "freezes" and ceases to provide movement as stimuli, the snake looses contact with it. Movement is then a cue for predators to recognize and detect preys (Curio E 1987[48]), so immobility may function as a defense behavior partially by making harder for the predator to locate the prey. Given its prevalence as a defense behavior it is then possible that animals in social groups are able to detect the onset of freezing in con and heterospecifics, and perceive it as a danger signal. Although this signal is not as specific as an alarm call or a wing whistle, it can be a widespread signal used by different species in the same environment when an approaching predator is detected. The findings in this work can therefore provide a framework to study the display of defense behaviors triggered by an acoustic cue that has an ecological meaning and may be used by different species.

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