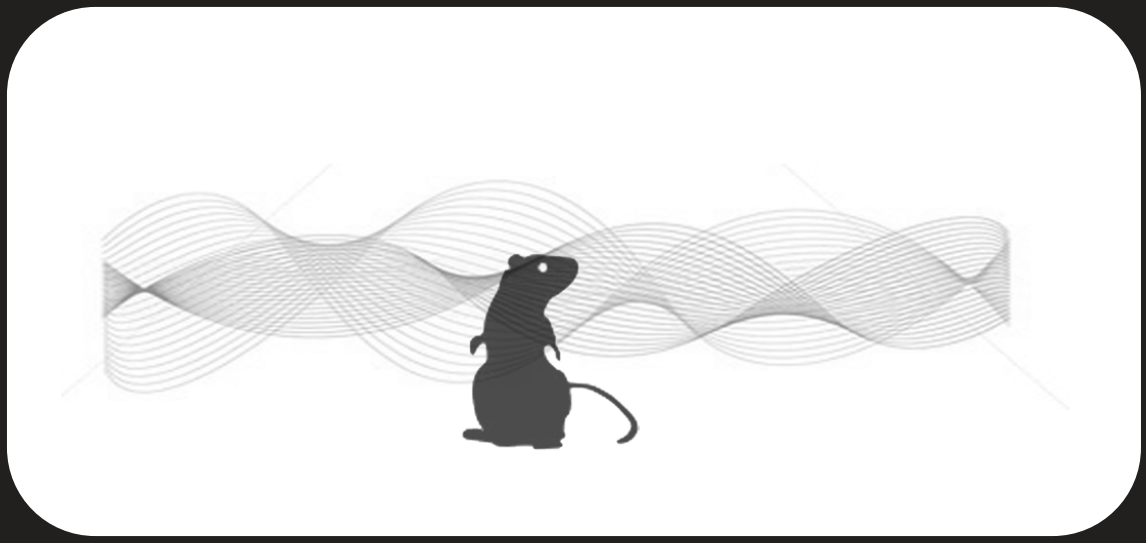
Neural mechanisms of stimulus generalization in auditory fear conditioning

Raquel A.G. Antunes



Dissertation presented to obtain the Ph.D degree in Neuroscience

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
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Knowledge Creation

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Research work coordinated by:
Marta Moita, PhD

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To my parents

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Resumo

O medo é uma resposta fisiológica com um forte impacto na sobrevivência e adaptação. Grandes progressos têm vindo a ser alcançados na compreensão dos mecanismos de aprendizagem de medo, fundamentalmente através do condicionamento da resposta de medo a estímulos auditivos. Neste paradigma comportamental um som inicialmente neutro (estímulo condicionado, EC) adquire propriedades aversivas após a sua associação a um estímulo aversivo (e.g. choque; estímulo não condicionado, ENC). Assim, após o condicionamento, o ENC desencadeia respostas condicionadas originalmente despoletadas pelo estímulo aversivo.

A amígdala tem sido identificada como um substracto neuronal relevante para a aprendizagem associativa da resposta de medo, sendo apontada como o local onde ocorre a associação EC-ENC. A informação auditiva converge na amígdala através de duas vias: directamente através do tálamo auditivo, ou indirectamente através de projecções tálamo-cortex-amígdala. Em virtude desta segregação de *inputs* foi proposta uma hipótese que defende a existência de uma "via superior" e uma "via inferior". Segundo esta hipótese, a via cortical ("via superior") é essencial para a discriminação perceptiva entre sons, enquanto que a via talâmica ("via inferior") transmite informação auditiva de uma forma mais rápida mas menos precisa.

O presente trabalho visava fundamentalmente testar esta hipótese para a qual, apesar de largamente aceite, poucas evidências têm sido apresentadas. O condicionamento da resposta de medo a estímulos auditivos foi usado como paradigma comportamental, e a hipótese da via superior/via inferior foi a base teórica para a

identificaçao dos substratos neuronais da discriminaçao auditiva na aprendizagem das respostas de medo.

Vários autores têm vindo a demonstrar que ambas as vias auditivas intervêm na aprendizagem da resposta de medo a estímulos auditivos, sendo que cada uma das vias é suficiente, por si só, para suportar a aquisição da resposta condicionada. Contudo, segundo um estudo recente, o córtex auditivo parece ser necessário para a expressão da memória após a aprendizagem de medo, o que, a confirmar-se, inviabilizaria experiencias destinadas a testar o papel da via cortical na aprendizagem discriminativa. Por esse motivo, o presente trabalho foi precedido de um estudo preliminar destinado a clarificar o contributo da via cortical para a aprendizagem no cérebro intacto, através da realização de lesões pós-treino do córtex auditivo.

Não obstante, importa também considerar que as lesões do córtex auditivo, subjacentes às dificuldades de aprendizagem anteriormente reportadas, afectaram quer o córtex auditivo primário, quer o cortex secundário e associativo. Face ao efeito destas lesões de grande extensão, e uma vez que os núcleos talâmicos que projectam para a amígdala estão também reciprocamente interligados ao cortex auditivo (sobretudo cortex secundário e perirrinal), as lesões efectuadas no âmbito do presente trabalho foram limitadas ao córtex auditivo primário (A1), cuja conectividade com os núcleos talâmicos que projectam para a amígdala é reduzida. Procurou-se, assim, minimizar a interferência de possíveis efeitos resultantes de lesões corticais que afectassem simultaneamente ambas as vias auditivas, quer por interferirem com a modulação corticofugal quer por induzirem degeneração neuronal dos núcleos talâmicos da via directa.

Resultados preliminares mostram a normal expressão da memória de medo em ratos treinados num protocolo *standard* de AFC e aos quais foram efectuadas lesões pós-treino do A1. Estes resultados permitiram então estabelecer a base dos estudos realizados subsequentemente com vista a avaliar a contribuição do cortex auditivo para a especificidade da informação auditiva transmitida à amígdala.

O facto de ter sido observado um resultado contrastante ao das lesões corticais de maior extensão anteriormente reportadas sugere uma contribuição diferencial dos *inputs* auditivos, a qual se encontra, provavelmente, segregada ao nível das vias lemniscal (tonotópica) e não-lemniscal (não tonotópica) que constituem a segmentação funcional base do sistema auditivo. Portanto, no presente trabalho foi levantada a hipótese de que a via tonotópica (mas não a via não-tonotópica), suporta a resposta discriminativa de medo a estímulos auditivos.

O córtex primário e secundário diferem principalmente na selectividade da resposta auditiva. O córtex auditivo primário, tonotopicamente organizado e cujos neurónios são caracterizados por uma resposta altamente selectiva, constitui o último elemento da via lemniscal e recebe projecções talâmicas originadas principalmente na igualmente tonotópica divisão ventral do tálamo auditivo (MGv), o único núcleo do tálamo que não possui projecções directas para a amígdala. Pelo contrário, na via não-lemniscal, a divisão média do tálamo auditivo (MGm) constitui o principal *input* directo para a amígdala (apesar de possuir também eferentes difusamente distribuídos no córtex auditivo), e apresenta respostas multisensoriais e de baixa selectividade.

Assim, a divergência funcional e conectiva verificada entre os *inputs* talâmicos foi utilizada como base teórica para testar a hipótese da "via superior/via inferior". Para o efeito, foram realizadas lesões electrolíticas do MGv ou do MGm e testado o seu efeito na aquisição, expressão e extinção da memória adquirida através de um protocolo de condicionamento diferencial da resposta de medo. Neste protocolo foram utilizados duas frequências sonoras diferentes, sendo que um dos sons foi associado ao choque (EC+) e o outro não (EC-).

Os resultados presentemente reportados mostram que através de condicionamento diferencial com uma só apresentação de cada um dos estímulos (*single-trial training*), todos os grupos testados (controlo, lesão do MGv e lesão do MGm) adquiriram respostas generalizadas de medo a ambos os sons. Por sua vez, utilizando um treino com múltiplas apresentações dos estímulos (*multiple-trial training*) os controlos expressam uma resposta diferencial de medo ao CS+ e ao CS-, enquanto que os animais com qualquer uma das lesões não discriminam os dois sons.

Por outro lado, quando as lesões foram realizadas após o treino de condicionamento, apenas os ratos com lesão do MGm revelaram incapacidade de discriminação entre os dois sons, sendo que este grupo de animais demonstrou igualmente elevados níveis de imobilidade (*freezing*), quer ao CS+ quer ao CS-, mesmo após uma sessão de extinção. Portanto, apesar de ambas as vias auditivas serem necessárias para a aquisição de respostas discriminativas de medo, a expressão destas respostas depende unicamente da via talâmica, sendo que esta via parece ser importante para a discriminação através da supressão da resposta de medo a estímulos neutros.

De um modo geral, os resultados apresentados sugerem o papel do MGv como modulador da aquisição de respostas discriminativas a estímulos auditivos aversivos, enquanto que o MGm parece sustentar continuamente a discriminação auditiva através de uma regulação negativa das respostas de medo. Face aos resultados obtidos, novas hipóteses são presentemente levantadas e discutidas no âmbito da contribuição das vias lemniscal e não-lemniscal para a aprendizagem discriminativa. O MGv poderá ser importante para a aprendizagem discriminativa por facilitação da selectividade no córtex, aumentando o contraste entre o EC+ e o EC-. O MGm, por sua vez, poderá agir como facilitador da plasticidade no cortex, ou através de convergência com o *input* cortical na amígdala. Por outro lado, o papel do MGm poderá estar relacionado com a manutenção do tónus inibitório ou na modulação inibitória dos neurónios da amigdala. Os mecanismos propostos, não sendo mutuamente exclusivos, podem em conjunto contribuir para a normal aprendizagem e expressão discriminativa da resposta de medo.

Abstract

Fear is a physiological trait with a strong weight on survival and adaptation. Great progress has been made to understand the mechanisms of fear learning, mainly using auditory fear conditioning (AFC). In this behavioral paradigm, an initial neutral tone (conditioned stimulus, CS) acquires aversive predictive properties after successive pairings with a footshock (unconditioned stimulus, US) and comes to elicit responses characteristically elicited by threatening stimuli. In this behavioral paradigm, the amygdala has been identified has a key neural substrate for associative fear learning, and the site where unconditioned stimuli (US) and conditioned (CS) auditory stimuli come to be associated.

Auditory information may reach the amygdala either directly from the auditory thalamus or indirectly via thalamo-cortico-amygdala projections. The “high route/low route” hypothesis has thus been proposed, which claims that the cortical pathway (“high route”) is crucial for discrimination between fearful and neutral sounds, while the direct thalamic pathway (“low route”) provides a rapid but less accurate relay of auditory information to the amygdala. This hypothesis relies on the assumption that more complex processing requires cortical activity and that thalamic relay is faster then cortical transmission to the amygdala. The present work essentially aims at putting to test this largely accepted hypothesis. Auditory fear conditioning was used as the behavioral paradigm to unravel the possible functional explanation for the coexistence of two parallel auditory pathways converging into the amygdala, and the high

route/low route hypothesis was the working model for the identification of neuronal substrates of auditory discrimination.

Accumulating evidence has been showing that each one of the pathways alone is sufficient to support auditory fear conditioning. However, according to a recent study, the auditory cortex might be necessary for the recall of auditory fear learning, which would render impossible the task of testing the role of the cortical pathway in the recall of discriminative fear. Therefore, the present work was preceded by a preliminary task aimed at clarifying the involvement of the cortical pathway in AFC in the intact brain, by performing post-training lesions of the auditory cortex.

Moreover, as lesions underlying the previously reported learning impairments encompassed both primary, secondary and association cortices, and because thalamic nuclei projecting to the amygdala are reciprocally connected to the auditory cortex, we selectively lesioned the primary auditory cortex (A1) which has limited connectivity with thalamic nuclei projecting to the amygdala. Through this selective cortical lesion we hoped to minimize effects resulting from lesions simultaneously affecting both pathways to the amygdala, either due to interference with corticofugal modulation or due to induced neuronal degeneration of thalamic nuclei projecting to the amygdala. Preliminary data shows normal expression of fear memory in animals with post-training lesions of A1 and trained in a standard AFC protocol. These results thus settled the basis for the following studies aimed at testing the role of the auditory cortex in the accuracy of conveyed auditory information during fear learning.

Because primary and secondary auditory cortex mostly differ on their tuning properties, and because the redundancy in the auditory

inputs to the amygdala essentially relies on the segregation of inputs in the leminscal (tonotopic) and non-lemniscal (non-tonotopic) systems, we further hypothesized that the tonotopic, but not the non-tonotopic, pathway supports discriminative fear to auditory cues. In the lemniscal pathway, sharply tuned neurons in primary auditory cortex receive their main input from the ventral division of the medial geniculate nucleus (MGv), which is tonotopically organized, has narrowly tuned neurons and does not project directly to the amygdala. In contrast, in the non-lemniscal pathway, the medial division of medial geniculate nucleus (MGm) shows multisensory and non-tuned auditory responses and is the main direct input to the amygdala, although it also sends diffuse projections to auditory cortex.

We thus went further on testing the high/low route hypothesis by assessing the effect of electrolytic lesions of the MGv or MGm on the acquisition, expression and extinction of fear responses. A discriminative auditory fear conditioning protocol was used, where one tone was followed by shock (CS+) and another was not (CS-). Here we show that with single-trial conditioning all the tested groups (control, MGv- and MGm-lesioned rats) acquire non-discriminative fear of both the CS+ and the CS-. This redundancy in neuronal pathways involved in the acquisition of fear may guarantee self-preservation, even though in single trial learning the learned fear responses generalize to other auditory stimuli.

After multiple-trial conditioning, control rats discriminate between the CS+ and CS-, whereas MGv- and MGm-lesioned rats do not, meaning that discriminative fear learning requires the activity of the two co-existing pathways. On the other hand, post-training lesions of MGm, but not of MGv, lead to impaired expression of discriminative

fear. Thus, although for the acquisition of discriminative fear both the lemniscal and non-lemniscal auditory pathways seem to be necessary, the recall of discriminative fear memory seems to rely solely on the latter. Interestingly, MGm-lesioned rats display high levels of freezing to both the CS+ and CS- even after an extinction session to the CS+, suggesting that this pathway might be important for discriminative fear by suppressing freezing to the neutral cues.

Altogether the present findings point out a role for the MGv as a modulator of the acquisition of discriminative fear responses, while the MGm continuously holds up for auditory discrimination by negatively regulating fear responses. New testable hypothesis are presently put forward concerning the contribution of the lemniscal and non-lemniscal routes for the mechanisms of auditory discrimination learning. MGv may be important for discriminative learning by facilitating cortical re-tuning, which might enhance the contrast between CS+ and CS- evoked activity in AC. The MGm may impact on discrimination learning by induction or facilitation of plasticity in cortex or through convergence with cortical input onto the amygdala neurons. Alternatively, the role of MGm may rely on sustaining inhibitory tone in the amygdala, or in the inhibitory modulation of amygdala neurons, namely through stimulus-specific inhibitory control of interneurons or by interacting with the inhibitory network of the central nucleus. These possibilities are not mutually exclusive and may all contribute to normal learning and expression of discriminative fear.

CHAPTER I - Introduction

Fear is a vital response to physical threats, and it is the mechanism through which individuals protect themselves from perceived danger. Learning from aversive events is thus a key stone in adaptive behavior. Furthermore, several behavioral disorders entailing maladaptive fear responses result from abnormal processing of threat-related stimuli, as well as functional deficits in brain pathways underlying fear learning and memory (Grillon, 2002b).

Though defensive responses are crucial for survival, they also bear physiological costs. Therefore, optimized behavioral responses thus demand that stimuli of higher biological significance should be susceptible to preferential neuronal representation and accuracy in discrimination. And so, neuronal mechanisms must exist which allow differential responding to neutral and aversive stimuli.

To address this issue, in the present work we used auditory fear conditioning (AFC) as the behavioral paradigm to study discriminative fear learning. This paradigm was chosen because it has been robustly used over time, and the neuronal circuit underlying AFC is quite well characterized. An introduction to the neuronal circuitry of AFC, as well as an elucidation on the functioning and connectivity of the auditory system thus follow in this chapter.

I.I Learning to fear: auditory fear conditioning

Learning from biologically relevant aversive events has been demonstrated in a wide range of species (Domjan, 2005; McNally and Westbrook, 2006). The prevalence of this form of learning in natural systems suggests that it is an adaptive trait that occurs under natural circumstances and increases fitness. But even though fear can serve as an alert mechanism for the organism against threat, pathological states entailing maladaptive fear responses can persist and have a negative impact in everyday life (Grillon, 2002a, 2002b).

Much of the understanding of the neural systems mediating fear conditioning has been achieved through research on animals. Nevertheless, recent studies suggest that similar systems are involved in human fear conditioning (LaBar et al., 1995; LaBar and LeDoux, 1996; Grillon, 2002a, 2002b, 2008; Ledoux and Muller, 1997). Studies on the neural basis of fear and anxiety in animal models may thus shed some light on the mechanisms underlying the development of pathological states of fear and anxiety and impact on the development of therapies for such behavioral disorders.

One of the simplest experimental tools for studying fear and anxiety is classical fear conditioning, based on Ivan Pavlov's findings that a neutral stimulus can acquire affective properties due to an association with a biologically relevant stimulus (Pavlov, 1968). Auditory fear conditioning has become in the last decade one of the most widely used paradigms to study the neural mechanisms of memory formation as fear learning entails robust, long lasting memories, and it is conserved across species. According to this paradigm, an initially neutral tone (conditioned stimulus, CS) acquires

aversive predictive properties after being paired with an aversive footshock (unconditioned stimulus, US). The CS, by virtue of its relationship with the US, comes to elicit responses characteristically driven by threatening stimuli, including changes in heart rate and arterial blood pressure, somatomotor immobility (freezing), hypoalgesia and pupillary dilation (Fendt and Fanselow, 1999; LeDoux, 2000; Maren, 2001) (Fig. 1). Because these responses are not elicited by the CS before the CS-US association, they are referred to as learned or conditioned responses.

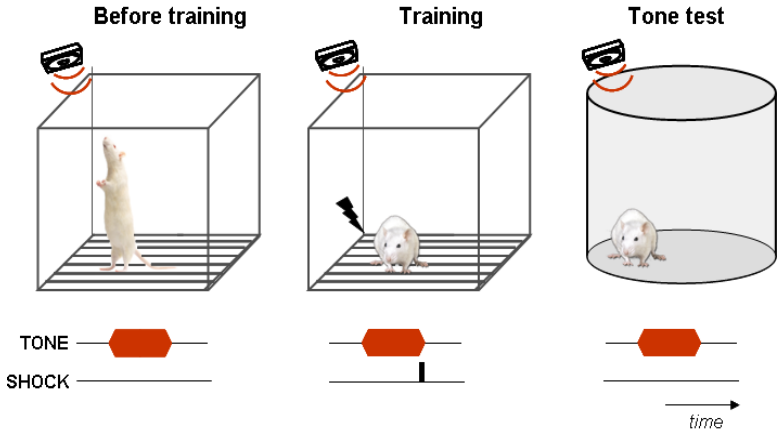

Figure 1 – Schematic representation of auditory fear conditioning. Before training, the tone (conditioned stimulus, CS) is a neutral stimulus, thus eliciting only exploratory behaviors. After the tone being pared with a mild footshock (unconditioned stimulus, US), when fear of the tone alone is tested, rats express fear responses typically displayed to the unconditioned stimulus.

In what concerns neuronal substrates of fear learning, extensive evidence, from genetic and pharmacological manipulations to electrophysiological recordings, points to the amygdala as a key structure for learning and recall of the CS-US association (Maren, 2001; Han et al., 2009; Koo et al., 2004; Rumpel et al., 2005; LeDoux, 2000; Fendt and Fanselow, 1999; Davis and Whalen, 2001). The amygdala is composed of several subnuclei (Sah et al., 2003; LeDoux, 2007), the most relevant for fear conditioning being the lateral (LA), basal (B) and accessory basal (AB) nuclei (many times referred to as "basolateral amygdala", BLA), which connects to the central nucleus (CE) (LeDoux, 2000; Pitkänen et al., 1995; Wilensky et al., 2006). Neurons in CE then signal to hypothalamic and brainstem regions that control the defensive and autonomic emotional responses to fear (LeDoux et al., 1988; Shi and Davis, 1999; Wilensky et al., 2006).

The amygdala receives sensory inputs from several brain areas, including the thalamus, the hippocampus and cerebral cortex (McDonald, 1998; Linke et al., 2000; Bordi and LeDoux, 1994a; Li et al., 1996; Romanski and LeDoux, 1993a; Iwata et al., 1986; Doron and Ledoux, 1999; Shin et al., 2006; Siguròsson et al., 2010), and it is widely believed that unimodal inputs enter the amygdala mainly through its lateral nucleus, as shown by anatomical, behavioral and physiological studies (LeDoux et al., 1990a, 1990b; Bordi and LeDoux, 1992; Romanski and LeDoux, 1992; Campeau and Davis, 1995a). In fact, cells in LA respond to both tones and footshock (Romanski et al., 1993), positioning the LA as a suitable locus for CS-US convergence in auditory fear conditioning.

Fear conditioning has been shown to enhance auditory responses in LA and long term potentiation (LTP), a possible cellular mechanism

underlying learning and memory formation, can also be induced in LA neurons (Quirk et al., 1995, 1997; Rogan et al., 1997; Rogan and LeDoux, 1995; Jung et al., 2010; Siguròsson et al., 2010; Ploski et al., 2010; Fourcaudot et al., 2009; Pan et al., 2009; Blair et al., 2001; Sah et al., 2008). Moreover, damage to LA has been shown to interfere with both the acquisition and expression of conditioned fear responses to auditory CSs (LeDoux et al., 1990a; Campeau and Davis, 1995a, 1995b; Nader et al., 2001; Amorapanth et al., 2000; Goosens and Maren, 2001; Maren et al., 1996; Maren, 1998, 1999), while blocking the AMPA receptor GluR1 trafficking in LA impairs LTP as well as fear conditioning (Rumpel et al., 2005). More importantly, the selective deletion of LA neurons recruited during learning blocks the expression of fear memory (Han et al., 2009). Altogether these data strongly suggest that activity in LA is necessary for formation of CS-US association.

However, even though the LA as been usually viewed as the locus for CS-US convergence, focus has been growing on the role of CE in auditory fear conditioning because this nucleus has been found to have the same characteristics that originally implicated the LA as a critical site for fear learning. The CE also receives afferent projections from the auditory cortex (McDonald, 1998) and the auditory thalamus (LeDoux et al., 1985b, 1985a; Turner and Herkenham, 1991), and it has been shown that these projections terminate in the lateral division of the central nucleus (CEl) (Linke et al., 2000; Turner and Herkenham, 1991; Pitkänen et al., 1995; McDonald, 1998; Jasmin et al., 1997; Wilensky et al., 2006), along with nociceptive information (Bernard et al., 1990; Jasmin et al., 1997; McDonald, 1998). In addition, the medial division of the central nucleus (CEm), the output

center of the CE, also receives projections from the auditory thalamus, namely the PIN (Turner and Herkenham, 1991; McDonald, 1998; Linke et al., 2000). Moreover, as for LA, high-frequency stimulation of thalamic inputs induces NMDA receptor-dependent LTP in the CE (Samson et al., 2005), the CE-lesions also impair the acquisition of fear conditioning (Goosens and Maren, 2001; Nader et al., 2001; Campeau and Davis, 1995b), and the inhibition of protein synthesis impairs the consolidation of fear memory (Wilensky et al., 2006). Altogether, these findings make the CE also well suited to integrate CS and US information during fear conditioning. Therefore, even though traditionally viewed as the major output structure, the central nucleus is also a potential critical site for fear learning.

During associative learning, nociceptive information about the US, ascending from the spinal chord, thus reaches the amygdala via LA or CE nuclei (Fig. 2-3). On the other hand, considering the traditionally accepted model, information about auditory CSs may reach the LA either directly from the auditory thalamus or indirectly via auditory cortex (Romanski and LeDoux, 1993a; Armony et al., 1995; McDonald, 1998; Linke et al., 2000; Li et al., 1996; Iwata et al., 1986; Doron and Ledoux, 1999; Shin et al., 2006; Siguròsson et al., 2010) (Fig. 2-3). The medial division of medial geniculate nucleus (MGm) and posterior intralaminar nucleus (PIN), which have multisensory neurons showing non-tuned auditory responses (except for high frequencies relating to social vocalizations) (Bordi and LeDoux, 1994b, 1994a), convey the main direct auditory input to the amygdala (LeDoux et al., 1985b, 1985a; Doron and Ledoux, 1999; Linke et al., 2000), although also sending diffuse projections to the auditory cortex (Kimura et al., 2003). Furthermore, the dorsal division of medial

geniculate nucleus (MGd) and the suprageniculate nucleus (SG) also directly project to the amygdala (Bordi and LeDoux, 1994a; Doron and Ledoux, 1999; Linke et al., 2000) (Fig. 7).

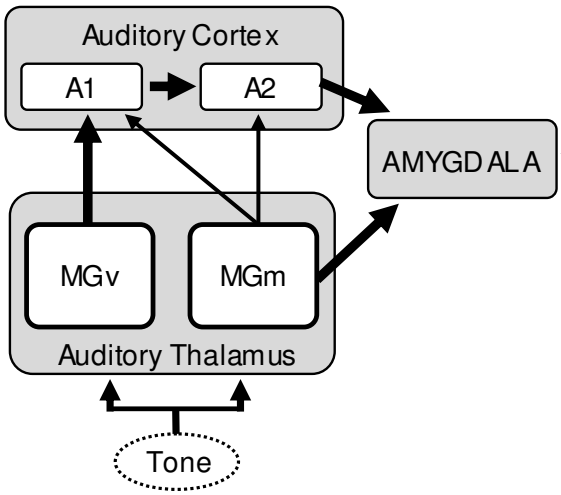

Figure 2 – Schematic showing the main direct (MGv) and indirect (MGm) auditory pathways to the amygdala. Thicker arrows represent major neuronal inputs. A1: primary auditory cortex; A2: secondary auditory cortex; MGv: ventral division of the medial geniculate nucleus; MGm: medial division of the medial geniculate nucleus.

Additionally, cortical projections to the amygdala originate in secondary auditory cortex (A2, also Te2/Te3) and in perirhinal cortex (PRh) (Doron and Ledoux, 1999; Romanski and LeDoux, 1993a, 1993b; McDonald, 1998). In the rat, it has also been reported that a

ventral portion of A1 (also Te1v), which appears to receive fewer projections from the MGv when compared to the remainder of A1, projects substantially to the LA (Doron and Ledoux, 1999; Romanski and LeDoux, 1993a, 1993b; McDonald, 1998). Considering both thalamo-amygdala and thlamo-cortical projections, MGv seems to project to BLA exclusively via cortical relay, while MGm/PIN and MGd project both directly and indirectly to the amygdala.

This segregation of inputs to the amygdala thus raises the question of what is the contribution of each pathway for fear learning. Accumulating evidence has implicated both the direct and indirect pathways of sound to the amygdala in AFC (Romanski and LeDoux, 1992; McKernan and Shinnick-Gallagher, 1997; Rutkowski and Weinberger, 2005; Boatman and Kim, 2006). For instance, glutamatergic transmission in the medial geniculate nucleus (including both MGm and MGv) is required for the recall of extinction memory (Orsini and Maren, 2009) and standard AFC has been shown to produce CS-driven frequency specific receptive field plasticity in the non-lemniscal MGm/PIN and MGd neurons (Edeline and Weinberger, 1991a, 1992), as well as in the lemniscal MGv neurons (Edeline and Weinberger, 1991b) and primary AC (Weinberger, 2007a, 2007b, 1998; Suga et al., 2002; Liu et al., 2007; Rutkowski and Weinberger, 2005; Edeline and Weinberger, 1993; Ma and Suga, 2009). It might thus be that both the AC and the MGm/PIN learn during auditory fear conditioning.

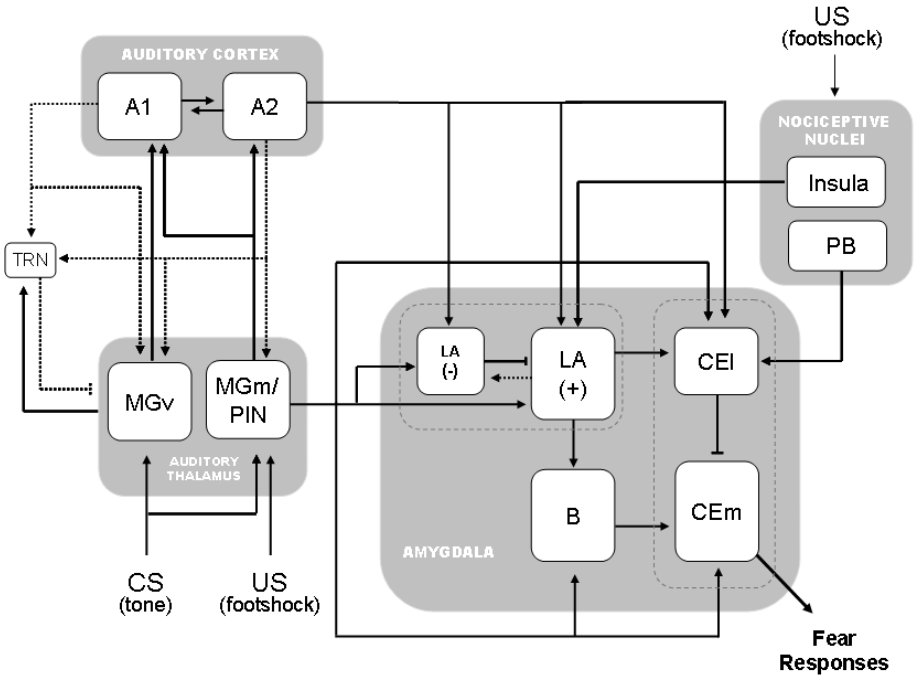

Figure 3 – Schematic showing the neuronal circuit underlying auditory fear learning. For simplicity, only the main thalamic inputs to the amygdala (MGm) and to the auditory cortex (MGv) are represented. For the whole set of thalamo-amygdala and thalamo-cortical projections see Fig. 6 and Fig. 7 in the following section of this chapter. Full lines represent feedforward projections and dashed lines represent feedback projections. Arrow-ended lines represent excitatory inputs, and dash-ended lines represent inhibitory inputs. MGv: ventral division of the medial geniculate nucleus; MGm: medial division of the medial geniculate nucleus; PIN: posterior intralaminar nucleus; A1: primary auditory cortex; A2: secondary auditory cortex; TRN: thalamic reticular nucleus; LA: lateral nucleus of the amygdala; (-): inhibitory interneurons; (+):principal neurons; B: basal nucleus of the amygdala; CEl: lateral subdivision of the central nucleus of the amygdala; CEm: medial subdivision of the central nucleus of the amygdala; PB: parabrachial nucleus.

On the other hand, several studies have also shown that the amygdala is under tight inhibitory control (Pan et al., 2009; Shaban et al., 2006; Shin et al., 2006; Bauer and LeDoux, 2004). A significant feedforward inhibition has been shown to arise from both thalamic and cortical projections to LA interneurons, even though feedforward inhibition from thalamic projections is stronger than that coming from cortex (Shin et al., 2006). Therefore, because local inhibitory networks have been shown to gate synaptic plasticity in the amygdala (Pan et al., 2009), cortical and thalamic inputs may also differently impact on fear learning by inhibitory mechanisms.

Overall, in what concerns the auditory inputs to the amygdala, each pathway alone seems to be sufficient for the acquisition of fear memory, as revealed by pre-training lesions of the whole AC (primary, secondary and perirhinal cortices) or MGm (Romanski and LeDoux, 1992). However, these same lesions, if performed after learning, seem to affect the recall of the auditory fear memory, with cortical lesions having the strongest impact on expression of fear responses (Boatman and Kim, 2006), while temporary inactivation of the auditory cortex renders animals behaviorally deaf (Talwar et al., 2001).

It has nevertheless been proposed that the indirect cortical pathway (“high route”) is crucial for discrimination between fearful and neutral sounds, while the direct thalamic pathway (“low route”) provides a rapid but less accurate relay of auditory information to the amygdala (LeDoux, 2000). This would allow a fast response in the case of danger, but the indirect route would stop the animal from generalizing its fear to a wide range of sounds. Accordingly, it has been shown that fear conditioning produces an increase in short-latency (10–20 ms) LA responses (Quirk et al., 1995), which is

consistent with the brief latency in transmission to the LA directly from the thalamus. In contrast, AC has been reported to develop plasticity slower than LA neurons (Quirk et al., 1997). It has also been shown that the earliest tone-evoked responses in MGm/PIN occur 7–9 ms after tone onset (Bordi and LeDoux, 1994a), while electrical stimulation of MGm/PIN produces responses in the amygdala about 5ms later (Clugnet and LeDoux, 1990), which is consistent with the short-latency LA responses induced by fear conditioning. On the other hand, previous physiological studies have identified a core region of the auditory cortex, the primary auditory cortex (A1), in which learning has been shown to expand the representational area of the CS based on the learned importance (Rutkowski and Weinberger, 2005; Weinberger, 2007b) (Fig. 11, section below).

Despite being traditionally accepted, little evidence supports the high/low route hypothesis. Moreover, auditory cortex lesions have in fact been shown not to affect the generalization across a gradient of tone frequencies (Armony et al., 1997), and a role for the thalamic pathway in differential fear responding has been previously suggested for conditioned bradycardia in rabbits (Jarrell et al., 1986, 1987), while increasing CREB levels in the MGm/PIN has been shown to result in broad auditory fear generalization (Han et al., 2008). Although the neural circuit underlying auditory fear conditioning has been intensively studied and its key players are now quite well characterized, further studies are thus still required to clarify the co-existence of parallel streams of information in the auditory fear circuit and elucidate individual contributions to associative learning of fear responses.

I.II The auditory system

The auditory system is a complex set of interconnected structures which allow the transduction of sound waves into neuronal signals so that ascending auditory information can be relayed into the central nervous system. The ability to acquire and process acoustic information about the environment, to localize and identify sound sources, communicate with conspecifics and heed auditory warnings, provides a selective advantage in an ever changing and challenging world. Hearing is a fundamental adaptation for survival and reproduction, whether it concerns communication within the species, warning against predators or locating preys, and it assumes particular significance in animals, namely in rodents, for which auditory signals are an important part of social interaction (Portfors, 2007).

The system underlying auditory processing is a multi-level assembly of interconnected structures. Two parallel pathways exist in the auditory system, an ascending auditory pathway, conveying auditory information from the organ of Corti to the auditory cortex (Fig. 4), and a descending stepwise projection from the cortex and all the way down to the cochlea (Paxinos, 2004) (Fig. 10), overall forming a plastic system with multiple loops.

The neural representation of sensory information undergoes a series of fundamental transformations as it ascends the central nervous system (Ehret, 1997; King et al., 2001; Pollak et al., 2003; Shamma and Micheyl, 2010). At the early stages of auditory processing, neurons of the auditory nerve and cochlear nucleus encode elemental properties of sound, such as amplitude, while at higher stages of processing unique patterns of convergence establish

neural receptive fields which are tuned to discrete acoustic features (Ehret, 1997; King et al., 2001; Pollak et al., 2003; Shamma and Micheyl, 2010).

A fundamental organizing principle of the auditory system is its tonotopic organization, preserved from the cochlea to the auditory cortex (Fig. 5), and resulting from different sensitivity of auditory neurons to frequency ranges (Ehret, 1997). The spectral sensitivity profile of a neuron has commonly been characterized as a frequency tuning curve, representing thresholds along the frequency domain (Ehret, 1997) (Fig. 5 and Fig. 8). Regarding frequency processing, numerous parallel and serial pathways converge in a common destination in the auditory system, the inferior colliculus (IC). From the IC and upward, the auditory pathway can be divided into a "core pathway" with tonotopic organization and very selective responding neurons (the lemniscal system), and a non-tonotopic and multisensory "belt pathway" (the non-lemniscal system) (Ehret, 1997; Paxinos, 2004). This functional segregation sets the basis for the differential flow of auditory information, and motivated the questions raised under the present work.

a) The ascending flow of auditory information

As sensory information ascends from the cochlea to the auditory cortex, it passes through several nuclei, each representing a neuronal substrate of auditory information. Neurons at each level in the auditory pathway exhibit complex and specific response properties which reflect sophisticated signal processing operations (Ehret, 1997; King et

al., 2001; Pollak et al., 2003; Shamma and Micheyl, 2010). Altogether, the auditory nuclei process the different components of auditory objects, like frequency, amplitude, distance or sound localization, thus representing the complexity of the auditory world (Ehret, 1997; King et al., 2001; Pollak et al., 2003; Shamma and Micheyl, 2010).

The peripheral sensory organ of the auditory system is the organ of Corti. Sound waves are transmitted mechanically through the outer and middle ear to the sensory hair cells of the organ of Corti, in the cochlear partition of the inner ear (Ehret, 1997; Paxinos, 2004). The mechanical energy underlying acoustic information is then transduced in the cochlea into bioelectrical energy in the receptor potentials of hair cells, and relayed to the cochlear nucleus via the auditory nerve fibers (Ehret, 1997). The frequency component of sounds is mapped along the cochlea basilar membrane and its overlying organ of Corti (Paxinos, 2004). The cochlear frequency map sets the basis for the tonotopic organization ultimately represented in the auditory cortex (Fig. 5 and Fig. 8). Signals of the cochlear nerve are then separated into a number of parallel ascending tracts, each with particular conduction velocities and relays. The cochlear nerve fibers, encoding stimulus frequency and intensity, terminate within the cochlear nucleus, which is the first nucleus of the central auditory pathway, and the first relay center in the ascending auditory pathway (Paxinos, 2004). The cochlear nucleus is particularly engaged in localization of sound sources. Cells in the ventral cochlear nucleus provide accurate information about the timing of acoustical stimuli, which is valuable for locating sound sources in the horizontal axis, while the dorsal nucleus is thought to participate in locating sound sources along the vertical axis (Kandel et al., 2000).

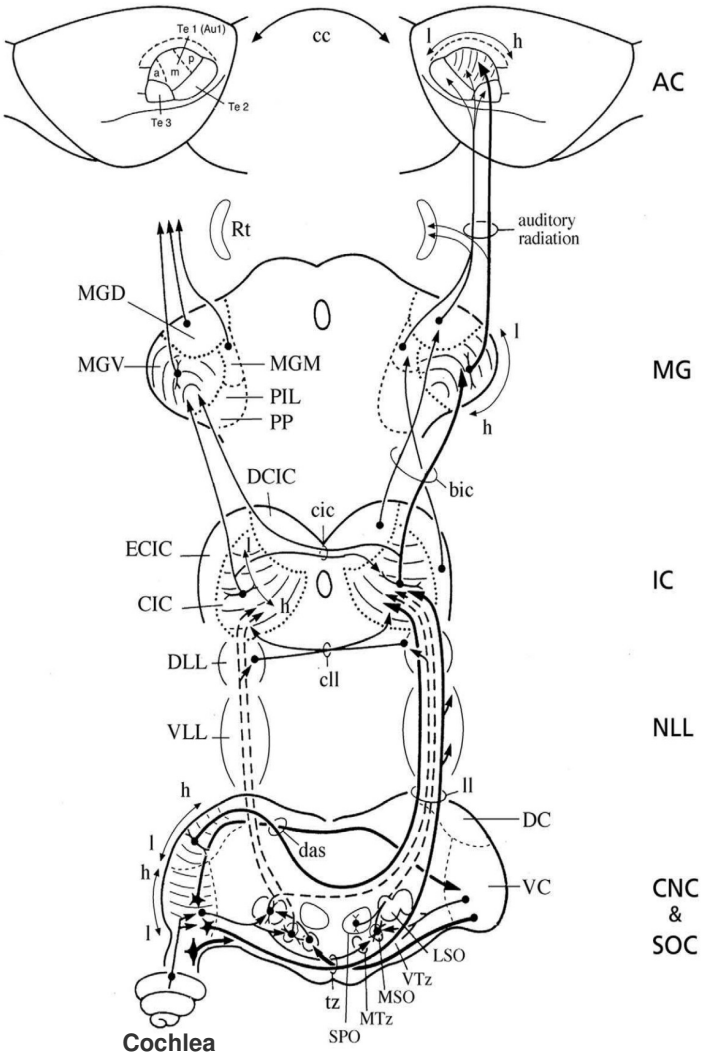

Figure 4- Ascending pathways of auditory information (adapted from Paxinos, 2004). Main auditory nuclei represented are: Cochlea (cochlea); Cochlear nuclear complex (CNC); Superior olivary complex (SOC); Nuclei of the lateral lemniscus (NLL); Inferior colliculus (IC); Medial geniculate body (MG) and Auditory cortex (AC).

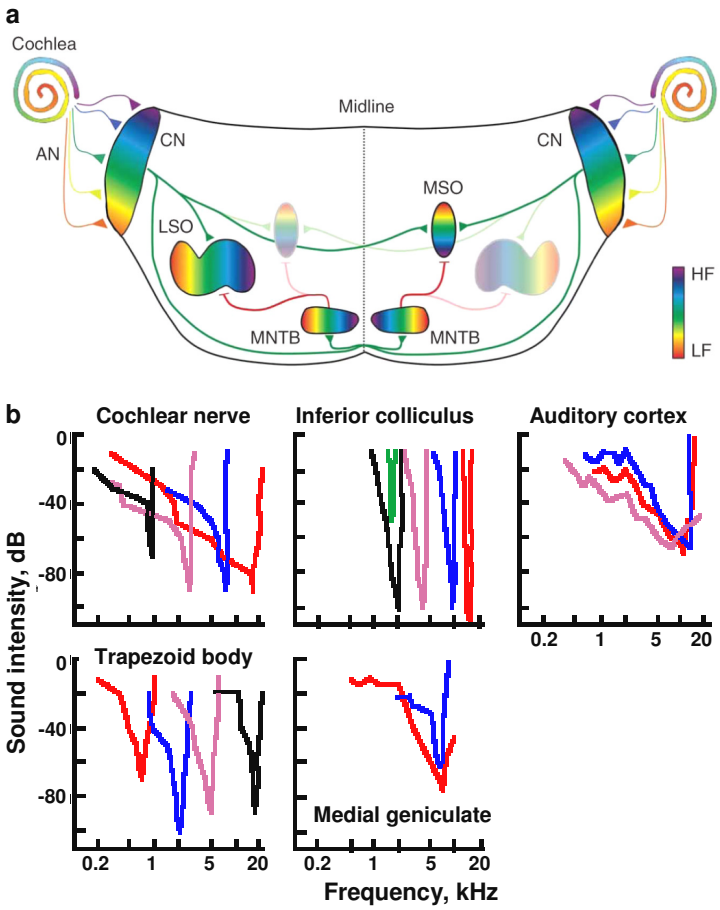

Figure 5 – Frequency selectivity in the auditory system of the mammalian brain. (a) Auditory frequency maps in auditory nuclei (adapted from Kandler et al., 2009). AN, auditory nerve; CN, cochlear nucleus; MSO: medial superior olive; LSO: lateral superior olive; MNTB: medial nucleus of the trapezoid body; HF, high frequency; LF, low frequency. (b) Tuning curves of neurons in different auditory nuclei (adapted from Mann, 2002). Tuning curves plot the sound intensity needed to increase the response of a neuron above spontaneous firing, and it represents the response area of the neuron. The frequency at which responses are elicited at the lowest intensity (tip of the V-shaped curve) is called characteristic frequency (CF).

There are three main output pathways of the cochlear nucleus: the superior olivary complex, the ventral acoustic stria and the lateral lemniscus (Ehret, 1997; Paxinos, 2004). The superior olivary complex is the first stage were robust convergence of auditory information from both ears takes place (Ehret, 1997). It consists of three nuclei, which are tonotopically organized: lateral superior olive (LSO), medial superior olive (MSO) and medial nucleus of the trapezoid body (MTZ) (Paxinos, 2004). In rodents, there is also a fourth nucleus, the superior paraolivary nucleus (SPO) (Paxinos, 1998, 2004; Saldaña and Berrebi, 2000). The MSO responds mainly to low frequencies while the LSO responds to all tonal frequencies (Paxinos, 2004). Therefore, species characterized by high upper frequency limits of their hearing range (e.g. rat), tend to have large LSO and small MSO. Furthermore, the MSO is engaged in localization of sound sources in the azimuthal axis through processing information about auditory delays of sound reaching each ear (interaural delays) and it contains a map of sound-source localization in the azimuth plane (Kandel et al., 2000). The LSO is also involved in the localization of sound sources, but through intensity cues, based on loudness of sound reaching each ear (Kandel et al., 2000).

The lateral lemniscus (LL), which is tonotopically organized (Merchán and Berbel, 1996; Paxinos, 2004), is a tract of axons in the brainstem that carries information about sound from the cochlear nucleus to various brainstem nuclei and ultimately to the contralateral inferior colliculus (Paxinos, 2004).

The ascending auditory tracts from cochlear nucleus, superior olivary complex and lateral lemniscus converge towards the auditory midbrain, in the inferior colliculus (IC). The IC has a key position as an

obligatory relay centre for most ascending auditory tracts (Fig. 4). Furthermore, it processes and integrates almost all ascending acoustic information from lower centres and determines the form in which information is conveyed to higher regions in the forebrain (Pollak et al., 2003). Neurons in the IC are involved in the integration of multi-modal sensory perception, processing of frequency- and amplitude-modulated sounds and sound localization, based on detection and representation of interaural timing and intensity differences (Kandel et al., 2000; Pollak et al., 2003; Paxinos, 2004).

The IC consists of a central nucleus (CIC), an external cortex (ECIC) and a dorsal cortex (DCIC), each with distinctive functional and connective properties (Faye-Lund and Osen, 1985). The CIC is characterized by a laminar structure which is the basis for its tonotopic organization (Faye-Lund and Osen, 1985; Malmierca et al., 1993, 1995). A narrow range of best frequencies is represented within each isofrequency lamina of the CIC, with neurons presenting V-shaped tuning curves, while single units in the DIC and ECIC have a clearly poorer tonal selectivity, characterized by broad and irregular tuning curves (Ehret, 1997). The CIC has ascending projections to the medial geniculate body (Peruzzi et al., 1997; Oliver et al., 1999) and well-developed commissural fiber systems (Malmierca et al., 2003). Moreover, the CIC projects in a strictly tonotopic manner to the ventral division of the medial geniculate body (Linke, 1999a; Peruzzi et al., 1997; Oliver et al., 1999).

The ECIC receives input from the cerebral cortex as well as from many non-auditory structures, and its neurons have been shown to respond not only to auditory but also to somatosensory input (for a review see Paxinos, 2004). The ECIC projects to the dorsal and

medial divisions of the medial geniculate body, while the DCIC projects only to the dorsal division of the medial geniculate body (Linke, 1999a; Peruzzi et al., 1997; Oliver et al., 1999).

Even though the majority of IC projecting neurons is glutamatergic, recent studies have found GABA-positive projection neurons in the CIC and a lower proportion in the DCIC and ECIC (Peruzzi et al., 1997). Because few GABAergic cells are present in the rat medial geniculate body (Winer and Larue, 1988), inhibitory inputs from the CIC may be important for bottom-up regulation of firing patterns in thalamic neurons.

From the inferior colliculus and upward, the auditory pathway can be divided into a tonotopic “core pathway” (the lemniscal system), and a non-tonotopic and multisensory “belt pathway” (the non-lemniscal system) (Ehret, 1997; Paxinos, 2004). The medial geniculate body (MGB), the main target for ascending projections from the inferior colliculus (Linke, 1999a; Peruzzi et al., 1997; Oliver et al., 1999), is the auditory centre of the thalamus. The MGB contains several divisions defined on the basis of cytoarchitecture and fiber connections (Linke et al., 2000; Linke and Schwegler, 2000; LeDoux et al., 1985b, 1985a; Ledoux et al., 1987; Winer et al., 1999, 1999). The ventral division (MGv) and the dorsal division (MGd) constitute its main core, while the thalamic nuclei surrounding the MGB in its adjacent posterior, medial, and rostral parts (the medial division of the MGB [MGm], the posterior intralaminar nucleus [PIN], the suprageniculate nucleus [SG] and the peripeduncular nucleus [PP]), integrate the caudal paralaminar nuclei (Linke and Schwegler, 2000; Linke, 1999a, 1999b). Due to response similarities between MGm and PIN neurons (Bordi and LeDoux,

1994b, 1994a), these nuclei are generally considered a single module (Armony et al., 1995).

The lemniscal core of the MGB is the tonotopically organized ventral division (MGv), which has narrowly tuned neurons (Bordi and LeDoux, 1994b, 1994a). In rat, MGv neurons show characteristic frequencies (CFs) along the whole tested spectrum (1-30kHz), while MGm and PIN neurons tend to show higher CFs (generally above 10Khz) (Bordi and LeDoux, 1994b). Another aspect of the MGV is the laminar arrangement of afferent fibers and principal neurons (Winer et al., 1999, 1999). In the rat, three subdivisions with different laminar patterns occur, namely the ventral nucleus, the ovoid nucleus, and the marginal zone (Winer et al., 1999). Furthermore, MGv neurons are organized in a gradient of frequencies which extends along the dorso-ventral axis, with lower frequencies being represented mainly in the dorsal part and higher frequencies mainly in the ventral part of the MGv (Bordi and LeDoux, 1994b). Studies in cats show that while the LV has the dorso-ventral gradient going from low to high frequencies, the OV is tonotopically organized but with a gradient from low to high frequencies along an axis going from its dorsomedial to its ventrolateral part (Ehret, 1997). However, no clear ventro-lateral gradient has been identified in the rat (Bordi and LeDoux, 1994b).

The main input to the MGV comes from the ipsilateral CIC (González-Hernández et al., 1991; Ledoux et al., 1987; Peruzzi et al., 1997), although a small projection from the contralateral CIC is also present (Paxinos, 2004). The ipsilateral input has excitatory and inhibitory components (Peruzzi et al., 1997), and many neurons in the MGV receive convergent excitatory and inhibitory input from the IC, though a significant number of neurons receive only excitatory inputs

and a few only inhibitory (Bartlett and Smith, 1999). MGV is also reciprocally connected with the tonotopically organized primary auditory cortex (Kimura et al., 2003, 2005; Hazama et al., 2004; Winer et al., 1999, 1999; Winer and Larue, 1987; Shi and Cassell, 1997), sending selective projections to layers III and IV of Te1 (Kimura et al., 2003) (Fig. 6).

The dorsal division of the MGB, part of the nonlemniscal system, is morphologically and anatomically complex, with five subnuclei whose function is poorly understood (Bordi and LeDoux, 1994b; Ehret, 1997; Paxinos, 2004). Like neurons in the MGv, it also receives excitatory and inhibitory inputs from the IC, excitatory inputs from the cortex, and inhibitory inputs from the reticular thalamic nucleus (Bartlett and Smith, 1999, 2002). However, unlike MGv, the MGD is not tonotopically organized (Winer et al., 1999) and is characterized by a large proportion of neurons which do not respond to acoustic stimuli or which are broadly tuned, with delayed responses that habituate faster (Bordi and LeDoux, 1994b). Furthermore, multimodal auditory-somatosensory responses are also found in this nucleus, mostly in its rostral part (Ledoux et al., 1987; Bordi and LeDoux, 1994a).

The major source of inputs to the MGD are the nonlemniscal parts of the inferior colliculus (Paxinos, 2004), though it also receives input from the spinal chord (Ledoux et al., 1987). On the other hand, MGd projects to all layers of primary auditory cortex, and to layers III and IV of the secondary auditory cortical areas (Kimura et al., 2003), to the insular cortex (Winer et al., 1999), and to the lateral nucleus of the amygdala (Doron and Ledoux, 1999; Linke et al., 2000) (Fig. 6 and Fig. 7).

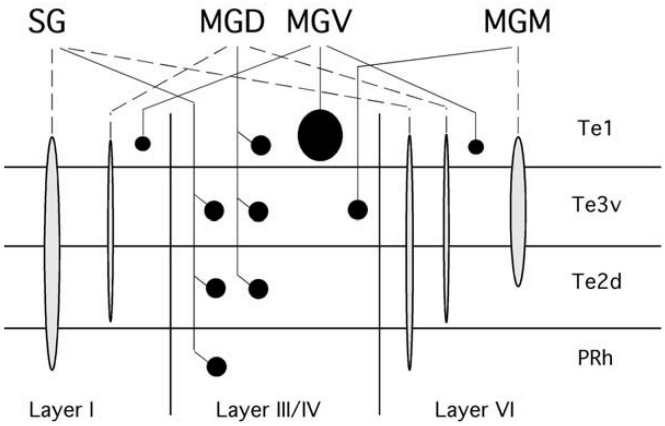

Figure 6 – Schematic representation of thalamocortical ascending projections (adapted from Kimura et al., 2003). Circles represent selective projections and extended terminations represent diffuse projections.

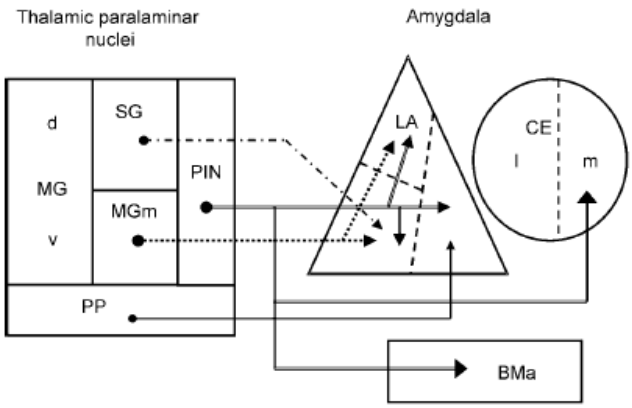

Figure 7 – Thalamic inputs to the amygdala subnuclei (adapted from Linke et al., 2000).

The medial division of the MGB (MGm) is also part of the non-lemniscal system of ascending auditory information. It has no tonotopic organization, though cells with higher CFs tend to be located more ventrally in the MGm (Bordi and LeDoux, 1994b). Contrary to the MGv, neurons in the MGm show broadly responding to a wider range of frequencies, though slightly narrower for higher frequencies, and tend to show higher characteristic frequencies, generally above 10Khz (Bordi and LeDoux, 1994b, 1994a). This nucleus sends diffuse projections to layer VI of all areas in the auditory cortex, though mainly targeting the secondary auditory cortex (areas Te2 and Te3) (Kimura et al., 2003) (Fig. 6). Moreover, MGm also projects to non-auditory regions and represents the main auditory input to the amygdala (LeDoux et al., 1985b, 1985a; Doron and Ledoux, 1999; Linke et al., 2000) (Fig. 7).

The PIN is part of the intralaminar and midline thalamic nuclei. Like in the MGm, PIN neurons tend to have higher CFs (generally above 16Khz), though generally more broadly tuned (Bordi and LeDoux, 1994b). Furthermore, despite its major input being auditory, and similarly to MGm, PIN neurons also respond to tactile, thermal, nociceptive, vestibular and visceral stimulation (Bordi and LeDoux, 1994a; Weinberger, 2010). In these nuclei, three types of neurons are found: those responding only to auditory stimuli, those responding to both auditory and somatosensory stimuli, and those responding only to somatosensory stimuli. Moreover, even unimodal somatosensory cells show increased responses with simultaneous presentation of somatosensory and auditory stimuli (Bordi and LeDoux, 1994a).This nucleus receives inputs from the IC (Ledoux et al., 1987; Linke, 1999a) and projects into layer I of the auditory cortex (Linke, 1999b;

Linke and Schwegler, 2000) (Fig. 6), also constituting one of the direct sensory inputs to the amygdala (Linke et al., 2000; Doron and Ledoux, 1999) (Fig. 7).

Additionally to the MGm and PIN neurons, the suprageniculate (SG) is also a site of auditory-somatonsensory convergence (Ledoux et al., 1987; Bordi and LeDoux, 1994a). Like in the MGm/PIN, neurons in the SG tend to have high CFs (generally above 16Khz), and are generally broadly tuned (Bordi and LeDoux, 1994a). This nucleus also represents one of direct sensory inputs to the amygdala (Bordi and LeDoux, 1994a; Linke et al., 2000) (Fig. 7).

Finally, the peripeduncular nucleus is a polymodal nucleus situated ventrally to the MGv which was also shown to project to the lateral and basal amygdala (Bordi and LeDoux, 1994a; Linke et al., 2000) (Fig. 7).

The auditory cortex constitutes the ending point in the ascending auditory pathways, particularly for the thalamic efferents (Kimura et al., 2003; Winer et al., 1999; Winer and Larue, 1987; Shi and Cassell, 1997). In the rat, the cortical map is generally categorized into three temporal areas: Te1 (core), Te2 and Te3 (belt areas), defined on the basis of the “gray level index” measured in Nissl staining (Zilles et al., 1980; Paxinos, 1998). Temporal area Te1 is considered to be the primary auditory cortex (A1) (Romanski and LeDoux, 1993a, 1993b), and areas Te2 and Te3 are considered the secondary cortices (Arnault and Roger, 1990; Paxinos, 2004). Furthermore, physiological studies have identified a core auditory field which has frequency selective neurons, surrounded by belt areas with less sharp frequency representation (Doron et al., 2002; Rutkowski et al., 2003) (Fig. 8a). In the rat, two tonotopically organized core fields, namely the primary (A1) and anterior (AAF) auditory fields, as well as three non-

tonotopically organized belt fields, namely the posterodorsal (PDB), dorsal (DB) and anterodorsal (ADB) belt fields have been identified (Doron et al., 2002; Rutkowski et al., 2003). Both A1 and AAF are located within Te1 (Doron et al., 2002; Rutkowski et al., 2003) (Fig. 8a).

Regarding frequency tuning, rat A1 neurons show a mean BW_{10dB} of approximately 1 octave (Kilgard et al., 2001; Rutkowski et al., 2003) (Fig. 8). Furthermore, mapping studies in A1 have found a low to high CF gradient that runs in the posterior to anterior direction (ranging from about 1 kHz to 50 KHz, according to the range of frequencies tested), with isofrequency lines oriented along the dorsoventral contour of the cortex, while AAF shows a reversal of frequency organization relative to A1 (Doron et al., 2002; Rutkowski et al., 2003) (Fig. 8a-b). When compared to neurons in A1, AAF neurons exhibit broader frequency tuning, as well as shorter first spike latencies and significantly higher thresholds (Doron et al., 2002; Rutkowski et al., 2003). Neurons in PDB, DB and ADB are characterized by strong responses to white noise and show either poor or no responses to pure tones (Doron et al., 2002; Rutkowski et al., 2003). The differences in response properties found between the core and belt fields may reflect a functional specificity in processing different features of auditory stimuli.

The auditory cortex is reciprocally connected with the MGB, although in the rat the reciprocity is not absolute (Winer and Larue, 1987). A1 receives selective ascending projections from the MGV (Romanski and LeDoux, 1993a; Winer et al., 1999; Kimura et al., 2003), though it also receives diffuse projections from the caudal parts of the MGD and the MGM (Kimura et al., 2003) (Fig. 6). Secondary

cortex receives projections from the MGd, MGM and SG (Romanski and LeDoux, 1993a; Winer et al., 1999; Kimura et al., 2003) (Fig. 6). In addition, the callosal fibers interconnect homotopic and heterotopic areas of the left and right auditory cortex (Rüttgers et al., 1990).

At the endpoint of the ascending flow of auditory information, the auditory cortex, namely its posterior and ventral regions (Te1v, Te3v and Te2c) and the interconnected perirhinal cortex (Romanski and LeDoux, 1993a), in conjunction with the subcortical projections from the auditory thalamic nuclei (MGm, PIN, MGd and SG) (Bordi and LeDoux, 1994b, 1994a; Linke et al., 2000), are further on route to convey processed auditory information to the amygdala, thereby mediating emotional responses to auditory stimuli.

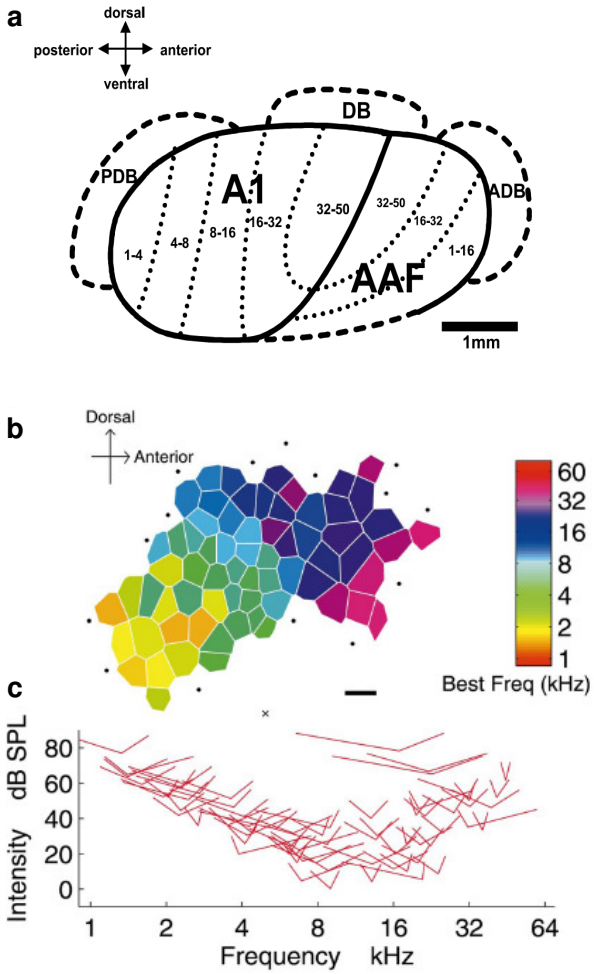

Figure 8 – Frequency selectivity in auditory cortex (adapted from Kilgard et al., 2001). (a) tonotopic map of core (primary auditory cortex [A1]) and belt regions (anterior [AAF], posterodorsal [PDB], dorsal [DB] and anterodorsal [ADB] auditory fields) of the auditory cortex. (b-c) example of a tonotopic map (b) and tuning curves (c) of primary AC from naïve rat. Color polygons represent characteristic frequency (CF), and the tip of V-shaped curves represent minimum threshold.

a) The descending flow of auditory information

Overall, the corticofugal auditory system forms multiple feedback loops along the descending auditory pathway. The corticothalamic projection forms the shortest auditory feedback loop, whereas the projection to cochlear hair cells through olivocochlear fibres forms the longest auditory feedback loop (Suga and Ma, 2003). Altogether, the corticofugal modulation loops seem to be important for the improvement and reorganization of subcortical auditory signal processing (Suga et al., 2000; Suga and Ma, 2003; Suga, 2008).

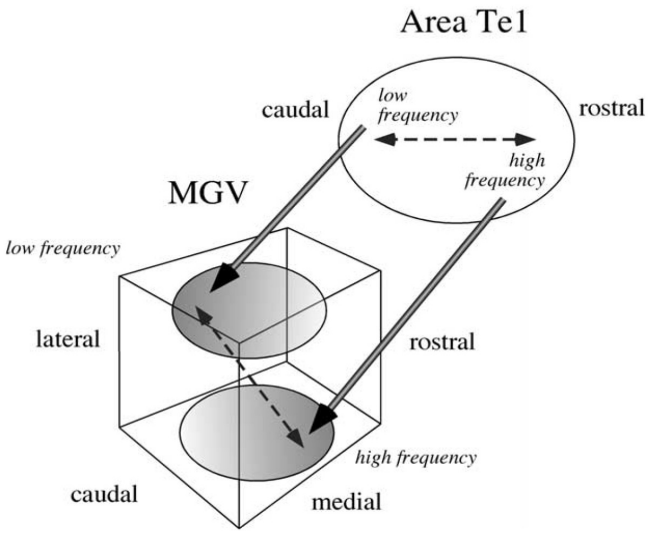

Figure 9 – Schematic representation of corticothalamic projections from primary auditory cortex (area Te1) to the MGv (adapted from Hazama et al., 2004).

Regarding topdown projections in the auditory system, the auditory cortex sends feedback projections to the MGB (Kimura et al., 2005; Hazama et al., 2004; Winer and Larue, 1987; Arnault and Roger, 1990; Shi and Cassell, 1997), the thalamic reticular nucleus (RTN) (Kimura et al., 2005; Zhang et al., 2008), the IC (Druga et al., 1997) as well as the cochlear nuclear complex (Weedman and Ryugo, 1996). In what concerns descending corticothalamic projections, it has been shown that the MGv receives the strongest cortical input, MGd receives moderate projections and MGm is the thalamic nucleus receiving the least cortical feedback (Winer and Larue, 1987). In particular, Te1 has been shown to project to MGv and MGd; Te2 projects to MGd, posterior paralaminar thalamic nuclei and sparsely to MGm; and Te3 projects to MGv, MGd, posterior paralaminar thalamic nuclei and sparsely to MGm (Winer and Larue, 1987; Arnault and Roger, 1990; Shi and Cassell, 1997; Kimura et al., 2005). The high-to-low frequency gradient on the primary auditory cortex has also been shown to give rise to corticothalamic projections targeting the MGV in a tridimensional arrangement, so that higher frequencies are mainly represented in the ventral-medial-rostral plane of the nucleus, and the lower frequencies are mainly represented in its dorso-latero-posterior plane (Hazama et al., 2004) (Fig. 9).

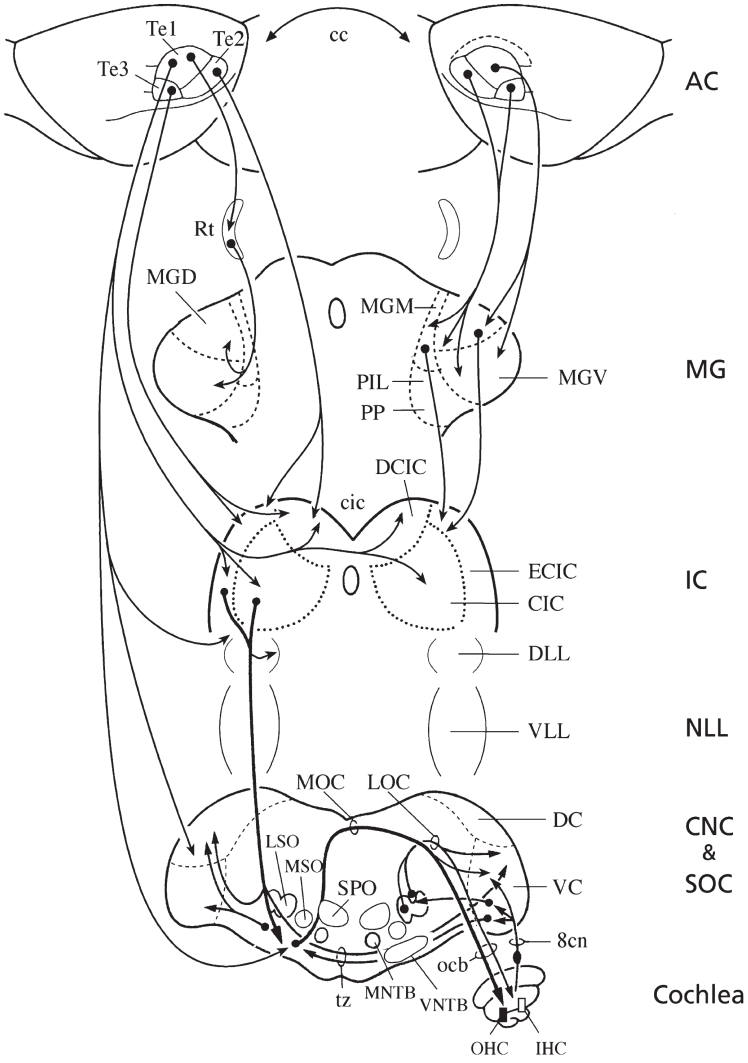

Figure 10 – Descending pathways of auditory information (adapted from Paxinos, 2004). Main auditory nuclei here represented are: Cochlea (cochlea); Cochlear nuclear complex (CNC); Superior olivary complex (SOC); Nuclei of the lateral lemniscus (NLL); Inferior colliculus (IC); Medial geniculate body (MG); Auditory cortex (AC).

Corticofugal inhibition of MGB neurons acts likely via feedback projections from the RTN. Placed between the reciprocal thalamo-cortical projections (Kimura et al., 2005; Zhang et al., 2008), the RTN consists on a sheet of GABAergic cells situated along the rostral and lateral surface of the dorsal thalamus (Guillery et al., 1998), and it receives ascending projections from all the thalamic nuclei (Paxinos, 2004; Kimura et al., 2005). This inhibitory pathway has been shown to play a role in the tonotopic control of frequency tuning in thalamic neurons (Cotillon-Williams et al., 2008).

Further descending in the auditory pathway, the dorsal nucleus of the IC has been shown to receive its inputs largely from the auditory cortex (Saldaña et al., 1996). The neocortical terminals make a tonotopic banded pattern like that of the ascending projections to the CIC (Saldaña et al., 1996; Druga et al., 1997). On the other hand, the external cortex receives inputs from the cerebral cortex, the medial geniculate body as well as from many non-auditory structures (Paxinos, 2004). Finally, descending projections from the IC target the superior olivary complex and the cochlear nuclear complex (Paxinos, 2004), thus bringing back processed auditory signals to the receiving organ in the auditory system.

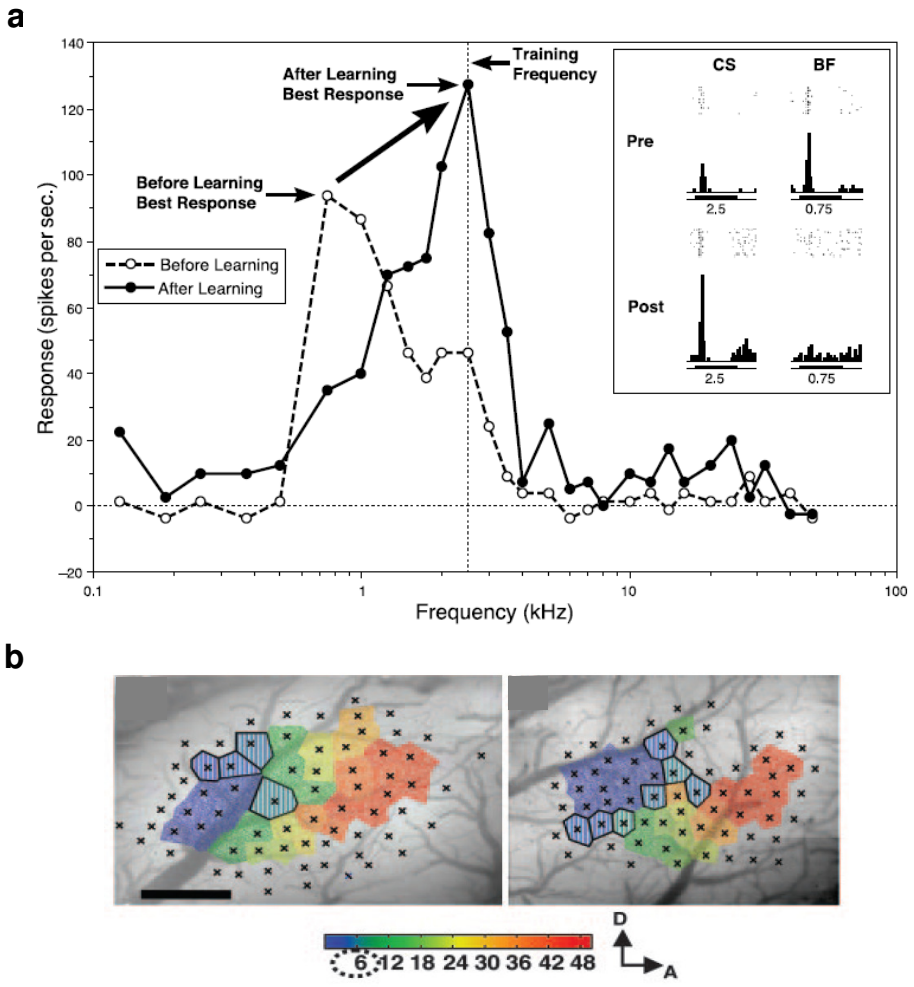

Figure 11 – Receptive field plasticity in the AC. (a) CS-specific tuning shift of a single cell in AC of guinea pig, resulting from auditory fear conditioning (adapted from Weinberger, 2007a). After conditioning, responses to the CS frequency increase and become the new characteristic frequency (CF). (b) tonotopic map of A1 in a naïve rat (left), and in a rat trained with 6KHz CS (right) (adapted from Rutkowski and Weinberger, 2005). Colored polygons indicate the estimated A1 area representing the CF according to the color bar shown below the maps.

Corticofugal modulation occurs for different types of subcortical neurons and is multiparametric (Suga et al., 2000; Suga and Ma, 2003; Suga, 2008). This system sharpens and shifts tuning curves of subcortical neurons in the frequency, amplitude, time and spatial domains and plays a key role in the reorganization of the auditory system according to auditory experience (Suga et al., 2000; Suga and Ma, 2003; Suga, 2008). In other words, the corticofugal auditory system improves and adjusts cortical input for auditory signal processing.

The response properties of neurons and the sensory maps in the auditory system can be changed by auditory learning. Cells in both the thalamo–amygdala and thalamo–cortico–amygdala pathways retune their frequency receptive fields, increasing responding to the CS frequency during conditioning, while decreasing responding to other frequencies, including the original best frequency (BFs) of the neuron (Edeline and Weinberger, 1991a, 1992). Furthermore, learning has been shown to expand the representational area of the CS in the tonotopic map of A1, and the learned importance of sound is encoded based on its representational size (Rutkowski and Weinberger, 2005; Weinberger, 2007b) (Fig. 11).

However, the precision of this frequency retuning is region-specific. Cells in the MGv, like in A1, typically develop a single sharp peaked frequency curve centered near the CS frequency, while the more broadly tuned cells in the MGm develop a generalized multi-peaked curve that responds to many tone frequencies (Edeline and Weinberger, 1991a, 1991b, 1992, 1993). On the other hand, the BF shift lasts at least 8 weeks in the AC (Weinberger et al., 1993) and less than 1 hour in the MGv (Edeline and Weinberger, 1991b).

Although sensory afferents certainly establish the basic receptive field properties of auditory neurons, increasing evidence indicates that feedback from the cortex plays a crucial role in shaping subcortical responses. For instance, cortical activation has been shown to shift the frequency tuning of unmatched CN neurons toward those of the activated cortical neurons (Luo et al., 2008). In the bat, when cortical neurons tuned to a specific frequency are inactivated, the auditory responses of subcortical neurons tuned to the same frequency are reduced (Zhang et al., 1997). Moreover, the responses of other subcortical neurons tuned to different frequencies are increased and their preferred frequencies are shifted towards that of the inactivated cortical neurons (Zhang et al., 1997).

The corticofugal system thus mediates a positive feedback which sharpens and adjusts the tuning of neurons at earlier stages in the auditory processing pathway, and is therefore expected to play a particularly important role in reorganizing the auditory system according to behavioral relevance of sounds.

I.III Goals

The main question underlying the present work concerns the understanding of how animals learn to differentially respond to fearful and neutral sensory stimuli. Because adaptive fear responses are a fundamental tool for survival, we aimed at looking for the neuronal network of fear to unravel how fear responses are fine-tuned according to the value of the stimuli.

Auditory fear conditioning was used as the behavioral paradigm to unravel the possible functional explanation for the coexistence of two parallel auditory pathways converging into the amygdala, and the high route/low route hypothesis was the working model for the identification of neuronal substrates of auditory discrimination. However, because it has been shown that the ascending auditory information on route to the cortex is segregated in two parallel streams, so that the redundancy in the auditory inputs to the amygdala essentially relies on the structural and functional segregation of inputs in the leminscal (tonotopic) and non-lemniscal (non-tonotopic) systems, we hypothesized that the tonotopic, but not the non-tonotopic, pathway supports discriminative fear to auditory cues.

Two main goals guided the present work, both explored in detail in chapter III:

1) Testing the role of the two parallel auditory streams in the acquisition of discriminative memory, by performing pre-training selective lesions of either lemniscal or non-lemniscal thalamic nuclei.

2) Testing the role of the two parallel auditory streams in the expression of discriminative memory, by performing post-training selective lesions of either lemniscal or non-lemniscal thalamic nuclei.

We thus aimed at testing the role of each auditory pathway based on the segregation of inputs at the thalamic level. However, previous studies have shown that the auditory cortex might be necessary for the recall of auditory fear learning, which would render the task of testing the role of the cortical pathway in the recall of discriminative fear impossible. Therefore, the present work was preceded by a preliminary task aimed at clarifying the involvement of the cortical pathway in AFC in the intact brain, by performing post-training lesions of the tuned primary auditory cortex.

CHAPTER II - The role of primary auditory cortex in auditory fear conditioning

Acknowledgements

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Summary

In auditory fear conditioning (AFC) information about auditory CSs reaches the amygdala, a key neural substrate for fear learning, either directly from the auditory thalamus or indirectly via auditory cortex (Fig. 2-3). A model for the functional role of these two pathways has been put forth based on the response properties of neurons along these two pathways, postulating that the cortical pathway ("high route") is crucial for discrimination between fearful and neutral sounds, while the direct thalamic pathway ("low route") provides a rapid but less accurate relay of auditory information. The hypothesis relies on the assumption that more complex processing requires cortical activity and thalamic relay is faster then cortical transmission to the amygdala.

Accumulating evidence has shown that both the direct and indirect pathways are implicated in AFC, although either one of the pathways alone seems to be sufficient to support auditory fear conditioning (Romanski and LeDoux, 1992). However, it has recently been shown that these same lesions, if performed after learning, seem to affect the recall of auditory fear memory, with cortical lesions having the strongest impact on expression of fear responses (Boatman and Kim, 2006), while temporary inactivation of the auditory cortex renders animals behaviorally deaf (Talwar et al., 2001). Because we were interested in the learning and recall of discriminative fear to test the high/low route hypothesis, a critical effect of AC-lesions on fear learning would thus hamper further experiments aimed at testing accuracy of AC-derived inputs. Lack of an effect of pre-training AC lesions does not imply that in the intact brain the AC is not used as the main pathway, in which case dissociation between a role of auditory

cortex in the expression of fear memory and a more specific role in discriminative fear learning would not be possible.

We thus started by re-examining the role of AC in the expression of fear memory by testing the effect of AC post-training lesions on the expression of fear memory. However, as lesions underlying the reported learning impairments encompassed the entire primary, secondary and perirhinal cortices, and because thalamic nuclei projecting to the amygdala are reciprocally connected to the auditory cortex, we thus hypothesized that previously reported learning impairments might result from lesions directly and indirectly simultaneously targeting both pathways to the amygdala. More importantly, because the redundancy of auditory inputs to the amygdala basically relies on the segregation between leminscal and non-lemniscal inputs (both targeting the AC), by doing such large cortical lesions both tuned and non-tuned auditory inputs to the amygdala were probably disrupted. Therefore, we decided to selectively lesion the frequency selective primary AC (A1), the lemniscal part of the AC, in order to minimize mixed effects of disrupting the two pathways because no significant connectivity between A1 and the main thalamic nuclei projecting to the amygdala seems to exist. Post-training cortical lesions were thus performed, and animals were trained in a standard auditory fear conditioning protocol to determine whether A1 is the main auditory pathway for learning, in which case an effect on retrieval of fear memory was expected.

Preliminary data shows normal expression of auditory fear memory in animals with post-training A1 lesions, thus setting the basis for further specific studies aimed at testing the accuracy of the cortical input during auditory fear learning.

Materials and Methods

Subjects

Subjects were naive male Sprague Dawley rats (300-450g) obtained from a commercial supplier (Harlan, Italy). After arrival animals were single housed in Plexiglas top filtered cages and maintained on a 12 hr light/dark cycle (lights on at 7:00 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 and surgical procedures were performed during the light phase of the cycle.

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Surgery

Aspiration lesions were performed under stereotaxic surgeries. Rats were anaesthetized with Sodium Pentobarbital (65mg/Kg) and given atropine (33mg/Kg). After the head being shaved, rats were place in a stereotaxic instrument with non-puncture ear bar. The scalp was incised and retracted and head position was adjusted to place bregma and lambda in the same horizontal plane. The skin and muscle above the skull was retracted and cranial holes were made above the lesion area. Using bregma as the reference point, two

lateral holes (one in each hemisphere) were drilled to expose auditory cortex surface, and then the primary AC tissue was removed by aspiration. The stereotaxic coordinates at the skull surface which delimitate the AC were: -3mm posterior to bregma, -6mm posterior to bregma. Sham lesions consisted of the same procedures but without any tissue removal by aspiration.

Thereafter sterile Vaseline was use to cover the holes in the skull and the skin was sutured. A single subcutaneous injection of the analgesic Buprenorphine (0.02mg/Kg) was given post-surgically. All the subjects were single-housed and allowed to recover from surgery for 7-10 days before any subsequent behavioral procedure.

Behavior setup

Two distinct environments (A and B) were used in this study. These two environments were located in the same procedure room and were used in a counterbalanced manner (i.e. the animals conditioned in A were tested in B and vice-versa). Both consisted of one conditioning chamber (model H10-11R-TC, Coulbourn Instruments) inside a high sound-attenuating cubicle lined with dark grey decoupling foam (model H10-24A Coulbourn Instruments). During training both chambers had a shock floor of metal bars (model H10-11R-TC-SF, Coulbourn Instruments), but during test sessions the floor in both chambers was covered by a painted acrylic floor. In order to minimize generalization between the two environments, several features of the environments differed. In box A, the ceiling and all four-side walls were made of clear Plexiglas and the sound-attenuating

cubicle was lined with yellow paper. The house light was in middle-top of the left wall and the speaker was placed outside the chamber, behind the right wall. On Box B the two sidewalls were made of polished sheet metal. The house light was red and placed in the top-back corner of the right wall and the speaker was behind the left wall. Furthermore, the boxes were cleaned with two different detergents.

The tones were produced by a sound generator (RM1, Tucker Davies Technologies) 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). A precision programmable shocker (model H13-16, Coulbourn Instruments) delivered the unconditioned stimulus footshock. A video camera mounted on the ceiling of each attenuating cubicle recorded the rats' behavior. A surveillance video acquisition system was used to store all video in hard disk for posterior off line scoring of freezing behavior by blind observers with timers.

Auditory Fear Conditioning

Intact animals were trained using a multiple-trial conditioning protocol. On day 1, rats were exposed to both the training and test contexts to avoid unspecific fear responses in the following days. On day 2 rats were trained in AFC protocol in one of the boxes, with 8 paired presentations of the tone CS (6.7 KHz, 20 sec) co-terminating with a footshock (0.5 mA, 1 sec), with an inter-trial interval of ± 3 min. The following day, animals were tested for fear of the tone CS (Test I) in a different box, with 2 presentations of the CS and an inter-trial

interval of ± 3min. Testing took place in a box differing from the training box in a number of cues (including texture, color and odor of the chamber, see above). Lesions were performed one day after the second training session. Rats were allowed to recover from surgery and then re-tested for their fear of the tone CS (Test II) one week after surgery.

Histology

At the end of each experiment all the animals were deeply anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 1% PBS salt solution followed by 10% formalin solution (Sigma). After this, brains were removed and stored in refrigerator in a 30% sucrose/formalin postfix solution until they sank (2 to 3 days). Then 40µm thick coronal sections covering the whole extent of the auditory cortex area were cut on a cryostat. Every third section was collected on coated slides and stained with cresyl violet. Sections were then examined in a light microscope to confirm location and extension of lesioned area.

Statistical Analysis

Freezing scores correspond to the duration of time spent freezing at specific time periods: before any CS was presented (20 sec baseline) and during each CS. Animals with a high baseline freezing score were excluded (above 50%, corresponding to abnormal values

which were defined by: freezing score > 3rd Quartile +1.5*[3rd Quartile – 1st Quartile]). Importantly, no differences in baseline freezing were found between groups (Mann-Whitney U Test: pre-lesion test: U= 7.50, p=0.743; post-lesion test: U=7.00, p=0.857). Because not all variables followed a normal distribution, homocedasticity is not met and due to the small sample size, we used non-parametric statistics. All analyses were performed using the statistical software XLSTAT, Microsoft®.

Results

Histology of Auditory Cortex lesions

The aspiration lesions consistently produced extensive damage to the primary auditory cortex, with minor spanning to the adjacent secondary cortical areas (Fig.12-13). Moreover, all animals with lesions whose depth extended medially to the hipoccampus were not included in the present study. After histological validation, a total of four rats were considered to have auditory cortex lesions reasonably confined to A1, and thus included in the analysis. Lesions in the present study were considerably smaller than those reported earlier in similar studies (Romanski and LeDoux, 1992; Boatman and Kim, 2006) and specifically restricted to A1, with minor lesions of adjacent secondary auditory cortex and complete sparing of the perirhinal cortex (Fig. 12c, 13c).

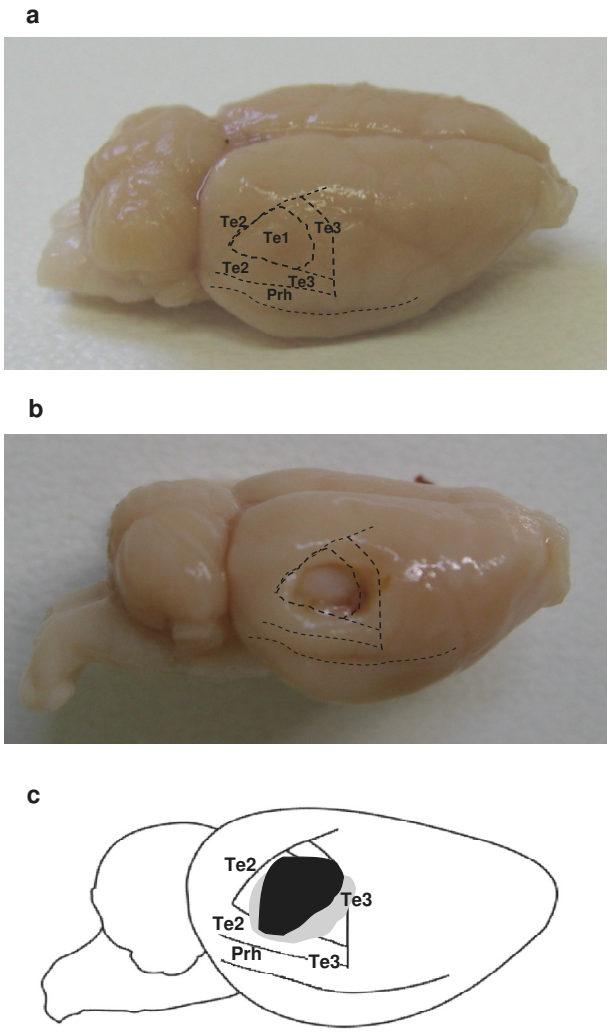

Figure 12 – Whole brain view of intact and AC-lesioned brains. Photographs show representative intact brain (a) and aspiration lesion of A1 (b). (c) Schematic showing extent of the largest (black) and smallest (light gray) aspiration lesions of AC. The parcellation of AC is based on that of Zilles et al. (1980): Te1, temporal area 1 (corresponding to primary AC); Te2/Te3, temporal areas 2 and 3 (corresponding to secondary AC); PRh, perirhinal cortex.

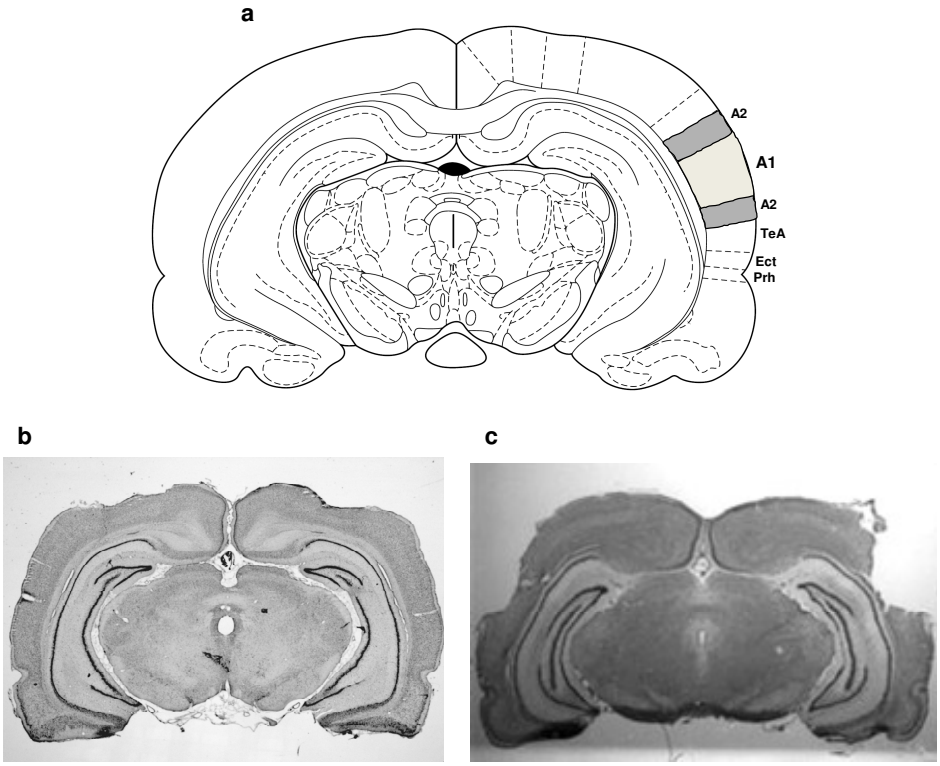

Figure 13 – Whole brain coronal sections showing auditory cortex. (a) Schematic of auditory cortex fields. (b) Photograph showing intact A1 of representative sham operated rat. (c) Photograph showing damaged A1 by aspiration lesion. A1: primary auditory cortex; A2: secondary auditory cortex; TeA: temporal association cortex; Prh: perirhinal cortex.

The primary auditory cortex is not required for the recall of auditory fear memory

Consistent with previous findings, Sham-operated rats acquired fear of the CS after auditory fear conditioning (Fig. 14a) and expressed it in the post-lesion test (Fig. 14b). Nevertheless, some degree of extinction seems to have occurred during the pre-lesion test, since freezing levels in the second test, post-lesion, were lower relative to the first test. In addition, increased pre-CS baseline freezing was observed in the post-lesion test, probably resulting from to second order conditioning deriving from the fact that both pre- and post-lesion tests were run in the same box.

Similarly, AC-lesioned rats showed intact acquisition of auditory fear responses (Fig. 14a) as well as expression of previously learned auditory fear responses in the post-lesion test comparable to that of sham animals (Fig. 14b). As with the Sham animals, extinction during the pre-lesion test session seems to have occurred, along with increased pre-CS baseline freezing. More importantly, no significant differences were observed between Sham and AC-lesioned animals, for either the pre-lesion test (Mann-Whitney test [pre-CS: U= 7.50, p=0.743; CS: U= 7.00, p=0.857; post-CS: U= 7.50, p=0.400]) or the post-lesion test (Mann-Whitney test [pre-CS: U= 7.00, p=0.857; CS: U= 7.50, p=0.743; post-CS: U= 8.00, p=0.629]). Interestingly, A1-lesioned rats consistently showed higher levels of freezing when compared to sham operated animals, even though only a small trend underlies this effect.

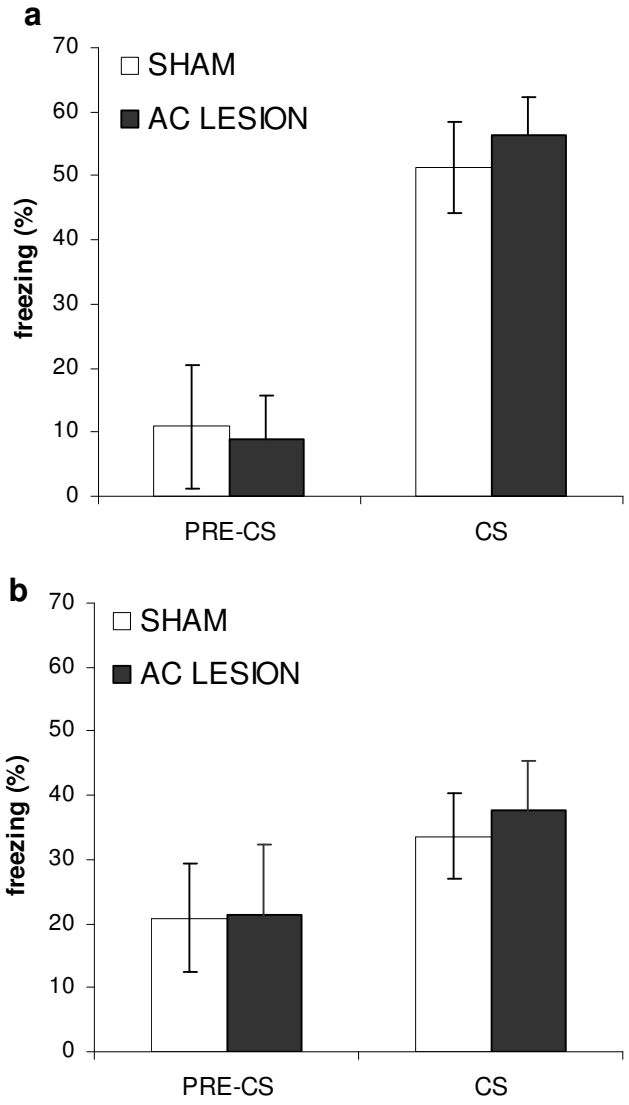

Figure 14 – Associative conditioned fear of tone CS during test trials: effect of primary auditory cortex lesions. Graphs show percentage of freezing during baseline pre-CS, CS and post-CS periods, in the tone test before (a) and after lesion (b). Data presented as mean±SEM for *n*=7 animals (Sham, *n*=3; A1, *n*=4). No significant differences between Sham and AC-lesioned groups were observed.

Discussion

The results of this preliminary experiment need to be interpreted with caution, due to the experimental design used, which was constrained by the available equipment. Notably, pre- and post-lesion tone tests were both performed on the same behavioral box, which might have increased pre-CS baseline freezing observed in the post-lesion test, probably resulting from second order conditioning to the test context. Nevertheless, even though pre-CS freezing might to some extent obscure CS-specific responses, no significant differences were observed between groups for either baseline or CS-driven freezing, so that the main question underlying this experiment regarding the role of primary AC in auditory fear learning could be answered.

The data reported here shows that post-training cortical lesions targeting mainly the core primary auditory field have no effect on the expression of previously learned freezing responses. In addition to previous data showing the sufficiency of the direct pathway in supporting the acquisition of AFC (Romanski and LeDoux, 1992), our work thus shows that the thalamic pathway is also sufficient for the recall of auditory fear memory.

However, these results contrast with the effect of auditory cortex lesions previously reported by Boatman and Kim (2006). According to these authors, post-training AC lesions completely abolished freezing responses to a conditioned tone, with freezing scores almost at 0%. The extent of the lesions seems to be a striking difference underlying our experiments. While we performed cortical lesions substantially confined to the core primary auditory field, lesions

performed by Boatman and Kim extended to the whole auditory cortex (A1 and A2) and adjacent perirhinal areas, which may account for our contrasting results.

Thalamic nuclei projecting to the amygdala (representing the "direct pathway") are also reciprocally connected to the auditory cortex, mainly to the secondary auditory cortex, either through direct corticothalamic excitatory projections (Kimura et al., 2003, 2005; Shi and Cassell, 1997; Winer and Larue, 1987; Arnault and Roger, 1990; Zhang et al., 2008; He, 2003) or through inhibitory descending projections via reticular thalamic neurons (Yu et al., 2004; Zhang et al., 2008; Cotillon-Williams et al., 2008; Suga and Ma, 2003). Therefore, by affecting corticofugal projections, whole extent AC lesions performed by Boatman and Kim might in fact be targeting both the direct and indirect pathways to the amygdala, which probably accounts for the results reported by these authors.

Furthermore, lesions performed by Boatman and Kim consisted of unilateral ablation of the whole auditory thalamus, further comprising contralateral ablation of the AC, thus completely disrupting auditory pathways on one hemisphere and further disrupting both pathways (at least partially, via MGm-AC connectivity) on the other one, which might also explain such low levels of freezing. More importantly, lesioning the entire AC may lead to significant degeneration of the auditory thalamus (Armony et al., 1997), thus resulting in the disruption of both the cortical and thalamic pathways to the amygdala.

Because weak connectivity between A1 and the main thalamic nuclei projecting to the amygdala has been reported (Kimura et al., 2003, 2005; Shi and Cassell, 1997; Winer and Larue, 1987; Arnault

and Roger, 1990; Zhang et al., 2008), by confining the lesions to A1, in the present experiment we minimized mixed effects resulting from disruption of the two pathways (due to thalamo-cortical interconnectivity), though some spreading of lesions over A2 was in some cases observed. Moreover, even if some degeneration of the thalamic nuclei occurred, it probably affected only the tuned thalamic nuclei projecting to A1 thus keeping intact the direct pathway to the amygdala.

Preliminary data reported here, by showing that A1 is not critically required for the expression of fear memory, thus allowed further studies to be conducted in order to elucidate the role of each pathway of sound to the amygdala in fear learning. Because the whole AC, but not A1 alone, is required for the expression of fear memory, and based on several studies identifying A1 as a neural substrate for physiological memory through retuning of receptive fields and encoding learned importance of sound based on its representational size (Ma and Suga, 2009; Weinberger, 2007a, 2007b; Suga et al., 2002; Liu et al., 2007; Rutkowski and Weinberger, 2005), new possibilities were left open in order to test the assumptions of the high/low hypothesis by studying the role of the A1 in accuracy-demanding learning tasks. Moreover, because primary and secondary auditory cortex mostly differ in their tuning properties, and considering the segregation of tuned and non-tuned auditory information at the subcortical level, further experiments were then designed to access the contribution of tuned information converging to A1 by selectively disrupting inputs ascending from the auditory thalamus.

CHAPTER III - Discriminative auditory fear learning requires both tuned and non-tuned auditory pathways to the amygdala

Acknowledgements

This work was supported by Fundação Champalimaud, Instituto Gulbenkian de Ciência and Fundacão para a Ciência e a Tecnologia, Portugal (POCTI/SAU-NEU/56935/2004). Raquel Antunes was supported by Fundação para a Ciência e a Tecnologia, grant SFRH/BD/27500/2006. Results on this chapter were fully obtained by the author.

Summary

The auditory system has two parallel streams converging into the amygdala which have both been implicated in auditory fear learning. The “high route/low route” hypothesis has traditionally been proposed, which claims that the cortical pathway (“high route”) is crucial for discrimination between fearful and neutral sounds, while the direct thalamic pathway (“low route”) provides a rapid but less accurate relay of auditory information to the amygdala.

The lemniscal stream has selective neurons that are tonotopically organized and is thought to be important for sound discrimination. The non-lemniscal stream has less selective neurons, which are not tonotopically organized, and is thought to be important for multimodal processing and for several forms of learning. Therefore we hypothesized that the lemniscal, but not the non-lemniscal, pathway supports discriminative fear to auditory cues.

In the lemniscal pathway, sharply tuned neurons in primary auditory cortex receive their main input from the ventral division of medial geniculate nucleus (MGv), which is tonotopically organized, has narrowly tuned neurons and does not project directly to the amygdala. In contrast, in the non-lemniscal pathway, the medial division of medial geniculate nucleus (MGm) has multisensory and non-tuned auditory responses and is the main direct input to the amygdale, although it also sends diffuse projections to auditory cortex (Fig. 2-3).

Therefore, to test the high route/low route hypothesis, we assessed the effect of electrolytic lesions to the MGv or MGm on the acquisition, expression and extinction of fear responses in

discriminative auditory fear conditioning, where one tone is followed by shock (CS+) and another is not (CS-). Since, in discriminative learning, animals first acquire generalized fear to both CS+ and CS- and discrimination between CSs is gradually learned with extended training (Pearce, 1997), we designed two training tasks that allow studying the mechanisms underlying the acquisition of generalized and discriminative fear, and tested the effect of MGm and MGv lesions on both training schemes.

Here we show that with single-trial conditioning control, MGv- and MGm-lesioned rats acquire non-discriminative fear of both the CS+ and the CS-, while after multiple-trial conditioning, control rats discriminate between the CS+ and CS-, whereas MGv- and MGm-lesioned rats do not. On the other hand, post-training lesions of MGm, but not MGv, lead to impaired expression of discriminative fear. Finally, MGm- but not MGv-lesioned rats display high levels of freezing to both the CS+ and CS-, even after an extinction session to the CS+. Altogether the present findings point out a role for the MGv as a modulator of the acquisition of discriminative fear responses, while the MGm continuously holds up for auditory discrimination by negatively regulating fear responses.

Materials and Methods

Subjects

Subjects were naive male Sprague Dawley rats (300-450g) obtained from a commercial supplier (Harlan, Italy). After arrival animals were single housed in Plexiglas top filtered cages and maintained on a 12 hr light/dark cycle (lights on at 7:00 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 and surgical procedures were performed during the light phase of the cycle.

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Surgery

Electrolytic lesions were performed under stereotaxic surgeries. Given the small dimension of the MGm nucleus and the fact that even small lesions had an effect on discrimination, performing excitotoxic lesions was not possible since just the injector track at the injection sites was sufficient to cause an effective lesion.

Rats were anaesthetized with Sodium Pentobarbital (65mg/Kg) and given atropine (33mg/Kg). The skin above the skull was retracted and cranial holes were made above the lesions area. Electrolytic

lesions were made using stainless steel electrodes (0.25 mm in diameter) insulated except for < 0.5 mm of the tip. A constant current source (Stimulus isolator, WPI) was used for all lesions (MGv lesions: 0.75 mA, 10sec; MGm lesions: 0.45 mA, 6sec). Stereotaxic coordinates relative to interaural zero according to Paxinos (1998) were used. Three penetration sites along the anterior posterior axis were used for both MGv and MGm lesions. For MGv lesions the coordinates were: anterior [+3.6mm, 3.2mm, 2.8mm anterior]; lateral [± 3.4mm, 3.5mm, 3.7mm]; ventral [+3.8mm, +3.7mm, +3.7mm]. For MGm lesions the coordinates were: anterior [+3.7mm, 3.2mm, 2.7mm anterior]; lateral [± 2.5mm, 2.6mm, 2.9mm]; ventral [+3.7mm, +3.7mm, +3.6mm]. In sham surgeries the electrode was placed 1.0 mm above the ventral coordinate without passing current. Once all penetrations were done the holes in the skull were covered with sterile Vaseline and the skin was sutured. A single subcutaneous injection of the analgesic Buprenorphine (0.02mg/Kg) was given post-surgically.

For the pre-training lesion experiments, animals were allowed to recover for one week after surgery before training begun. For the post-training lesion experiments, surgeries were performed 24 hours after the last training session. The animals were then allowed to recover for one week after which the discrimination test session took place.

Behavior

Two distinct environments (A and B) were used in this study. These two environments were located in the same procedure room and were used in a counterbalanced manner (i.e. the animals conditioned in A were tested in B and vice-versa). Both consisted of one conditioning chamber (model H10-11R-TC, Coulbourn Instruments) inside a high sound-attenuating cubicle lined with decoupling foam (sound isolation chamber, Action Automation and Controls, Inc.). During training, both chambers had a shock floor of metal bars (model H10-11R-TC-SF, Coulbourn Instruments) but during test sessions, the floor in both chambers was covered by a painted acrylic floor. In order to minimize generalization between the two environments, several features of the environments differed. In box A, the ceiling and all four-side walls were made of clear Plexiglas and the sound-attenuating cubicle was lined with white paper. The house light was in middle-top of the left wall and the speaker was placed outside the chamber, behind the right wall (Fig. 15, top boxes). On Box B the two sidewalls were made of polished sheet metal and the sound-attenuating cubicle was lined with black paper. The house light was red and placed in the top-back corner of the right wall and the speaker was behind the left wall (Fig. 15, bottom boxes). Furthermore, the boxes were cleaned with two different detergents.

The tones were produced by a sound generator (RM1, Tucker Davies Technologies) 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). A precision programmable shocker (model H13-

16, Coulbourn Instruments) delivered the unconditioned stimuli footshock. A video camera mounted on the ceiling of each attenuating cubicle recorded the rats' behavior. A surveillance video acquisition system was used to store all video in hard disk for posterior off line scoring of freezing behavior by blind observers with timers.

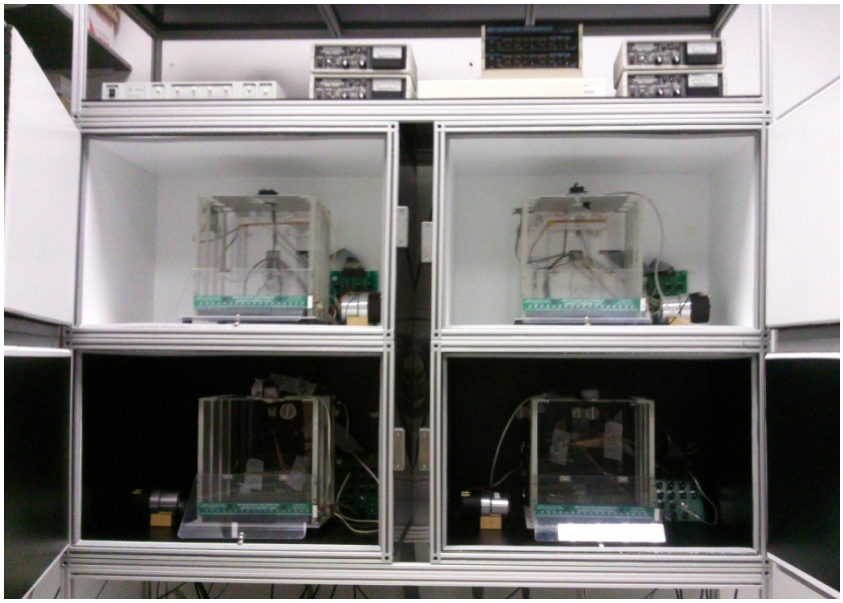

Figure 15 – Behavioral setup for auditory fear conditioning. Photograph shows conditioning boxes inside sound attenuating chambers. Different training/test contexts are shown: context A (2 top boxes) and context B (2 bottom boxes).

Experiment 1 – Role of MGv and MGm in acquisition of Auditory Fear Conditioning

Animals were subjected to one of two training protocols: single or multiple-trial conditioning (Fig. 16a, 17a). Single-trial conditioning consisted of one single presentation of the CS+ co-terminating with a footshock (0.5 mA, 0.5 sec), followed by a single presentation of the CS-, with an 180 sec inter-trial interval (ITI). Multiple-trial conditioning consisted of two sessions, each comprising 4 random presentations of the CS+, which co-terminated with a footshock (0.5 mA, 0.5 sec), and 4 random presentations of the CS-, with an average 180 sec ITI. In both protocols the CS+ was a 10 KHz pure tone (60 dB, 20 sec) and CS- was a 2 KHz pure tone (60 dB, 20 sec). These tone frequencies were chosen as they lie in the region of the auditory spectrum to which both neurons in amygdala and MGm show flat receptive fields, i.e. no discriminative firing in naïve animals can be observed. Neurons in these structures show selective firing for higher frequency ranges (Bordi and LeDoux, 1994a-b), which correspond to social ultrasonic vocalizations (USVs). As we are interested in general mechanisms of discrimination we chose to avoid frequencies that are close to the ones used in social communication. Furthermore, it has been shown that rats trained in a differential CS+/CS- AFC protocol tend to generalize their fear responses towards 22 KHz tones, which corresponds to the fundamental frequency of alarm calls (Bang et al., 2008). Thus, the frequency of the CS+ was chosen to be the 10 KHz frequency (which is closer to the 22 KHz principal frequency), to avoid biasing our results towards generalization, which may arise from responses to USVs and innate fear.

Rats were tested for their fear of the CS+ and CS- 24 hours after the last training session. The same testing protocol was used for rats subjected to the single and multiple-trial conditioning protocols. Testing took place in a box differing from the training box in a number of cues (including texture, color and odor of the chamber, see above). Three presentations of the CS- were followed by 3 presentations of the CS+, with a 5min ITI.

Experiment 2a –Role of MGv or MGm in the recall of discriminative Auditory Fear Conditioning

Intact animals were trained using the multiple-trial conditioning protocol of experiment 1. Lesions were performed one day after the last training session. Rats were allowed to recover from surgery and tested for their fear of the CS+ and CS- one week after surgery. Like in Experiment 1, testing took place in a different box and the same testing protocol was used (Fig 18a).

Experiment 2b – Role of MGv or MGm in the extinction of previously learned discriminative fear responses

For this experiment a subset of animals from the three groups (Sham, MGv and MGm lesion) of Experiment 2 was used. One day after the discrimination test these animals underwent an extinction session (10 presentations of the CS+) in the testing box. One day later freezing to the CS- and the CS+ was re-tested using the same testing

protocol as before (Fig. 18a). Thus, even though animals were conditioned with an intact brain, extinction training was performed in lesioned animals.

Histology

At the end of each experiment all the animals were deeply anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 1% PBS salt solution followed by 10% formalin solution (Sigma). After this, the brains were removed and stored in refrigerator in a 30% sucrose/formalin postfix solution until they sank (2 to 3 days). Then 40µm thick coronal sections covering the whole extent of the MGm or MGv areas were cut on a cryostat. Every third section was collected on coated slides and stained with cresyl violet. Sections were then examined in a light microscope to confirm location and extension of lesioned area.

For all experiments, from a total of 31 MGv-lesions performed, 16 were excluded, and from a total of 57 MGm-lesions performed, 37 were also excluded due to misplacement, small extension or lesions extending to the neighboring nuclei.

Statistical Analysis

Freezing scores correspond to the duration of time spent freezing at specific time periods: before any CS was presented (20 sec baseline) and during each CS+ and CS-. In all experiments freezing

scores during the CS+ and the CS- were normalized to the baseline (for each animal the difference between freezing during each CS and baseline was calculated), so that differences between freezing scores during the CS+ and CS- do not reflect individual differences in baseline fear. Animals with a high baseline freezing score were excluded (above 50%, corresponding to abnormal values which were defined by: freezing score > 3^{rd} Quartile +1.5*(3^{rd} Quartile – 1^{st} Quartile)). Importantly, no differences in baseline fear were found between groups in any of the experiments (see figures 16c, 17c and 18c; Kruskal-Wallis tests: experiment 1, single trial conditioning, $K_{(2)}$=3,06, p=0.22; experiment 1, multiple trial, $K_{(2)}$=3,73, p=0.16; experiment 2, $K_{(2)}$=1,12, p=0.57; experiment 3, $K_{(2)}$=1,89, p=0.39).

Because not all variables followed a normal distribution, homocedasticity is not met and due to the small sample size, we used non-parametric statistics. Discrimination was assessed by testing whether freezing evoked by the CS+ was higher than that triggered by the CS-. To this end, one-tailed Wilcoxon ranked signed tests, performed within groups, and Bonferroni corrected for multiple comparisons (critical value, α=0.017) were used. All analyses were performed using the statistical software XLSTAT, Microsoft®.

For comparing the effect of MGv lesions targeting different frequency ranges along the dorso-ventral axis on discrimination differential freezing (d; Cohen, 1988) was calculated as follows:

$$d = \frac{(\text{mean freezing to } CS^{+}) - (\text{mean freezing to } CS^{-})}{\sqrt{(SD_{CS+}{}^{2} + SD_{CS-}{}^{2})/2}} .$$

Results

MGv and MGm are both required for acquisition of discriminative Auditory Fear Conditioning

Consistent with previous findings, Sham animals acquired fear of the CS+ and generalized to the CS- after single-trial conditioning (Fig. 16b-c, no significant difference between freezing to the CS+ and the CS-, V=24.0 and p=0.23). Similarly, MGv- and MGm-lesioned rats showed intact acquisition of generalized fear responses after single-trial conditioning (Fig. 16b-c, no significant difference between CS+ and CS- elicited freezing was observed: V=16.0 and p=0.42, V=7.0 and p=0.50, respectively). Thus, both pathways are sufficient for single-trial conditioning, which entails conditioned fear generalized to the CS.

Multiple-trial conditioning lead to the acquisition of discriminative freezing in control animals (Fig. 17b-c, freezing to the CS+ was significantly higher than to the CS-, V=0.0 and p=0.0005 for the sham-lesioned group). In contrast, MGv-lesioned animals failed to discriminate between CS+ and CS- after multiple-trial conditioning (Fig. 17b-c, again freezing during the CS+ was not significantly different from freezing during the CS-, V=5.0 and p=0.08, note that the critical value α is 0.0167).

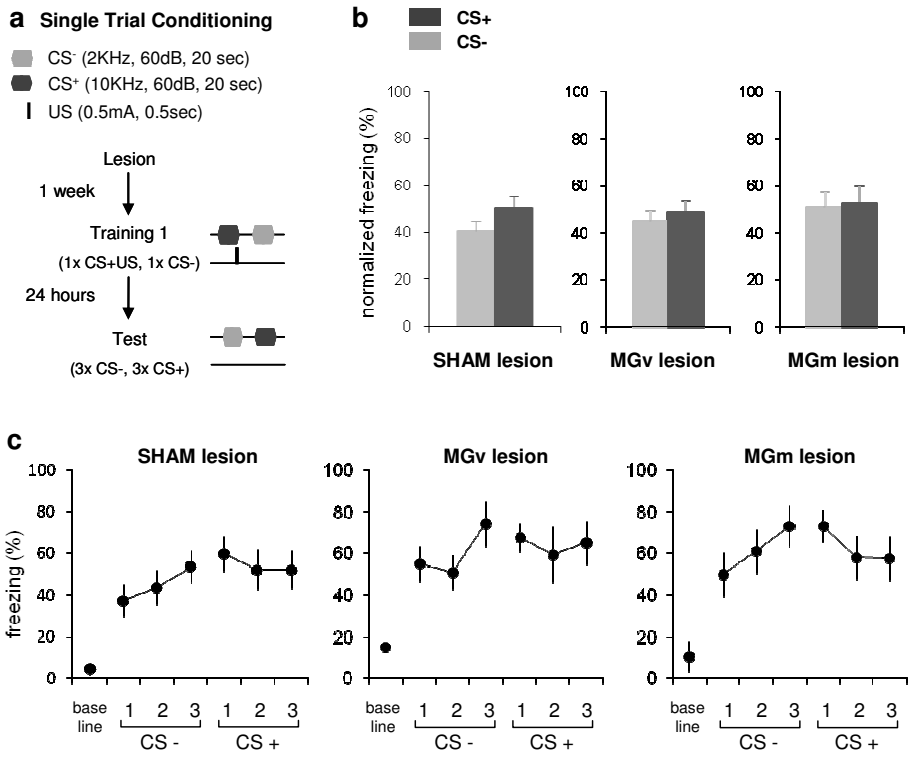

Figure 16 - Each auditory pathway to the amygdala is sufficient for the acquisition of generalized fear. (a) Schematic showing experimental protocol. (b) – (c) Freezing responses to CS+ and CS during discrimination test. Freezing responses are shown as average percent freezing normalized to baseline freezing levels (b) and as raw data, with percent time freezing during the baseline (first Pre-CS presentation), and each presentation of the CS+ and CS- (c). Data presented as mean±sem for n=24 animals (control N=11; MGv-lesion N=8; MGm lesion: N=5). Critical value: $p<0.0167$.

Additionally, we found that MGm-lesioned rats also failed to discriminate between CS+ and CS- after multiple-trial conditioning (Fig. 17b-c, no significant difference between freezing to the CS+ and CS was found, V=6.5 and p=0.45). Importantly, this result cannot be explained by a possible disruption of MGv projections that may cross MGm, as this would correspond to a combined lesion of MGv (fibers) and MGm (cell bodies), which should lead to impaired auditory evoked freezing (LeDoux et al., 1984).

Furthermore, with pre-training MGm-lesions we observed intermediate levels of freezing to both CSs. We understand this effect as resulting from the possibility that, when an animal cannot discriminate between the CS+ and the CS-, it will perceive all CS presentations as the same sound, but only half of these were paired with footshock. Thus, it should be equivalent to a partial reinforcement paradigm, so that both CSs become equally reinforced but at an intermediate level. In contrast, MGv lesions seem to result in impaired discrimination due to increased freezing to the CS- when compared to Sham animals. Therefore, even if through different mechanisms, both the lemniscal and the non-lemniscal pathways are required for intact discriminative fear acquisition.

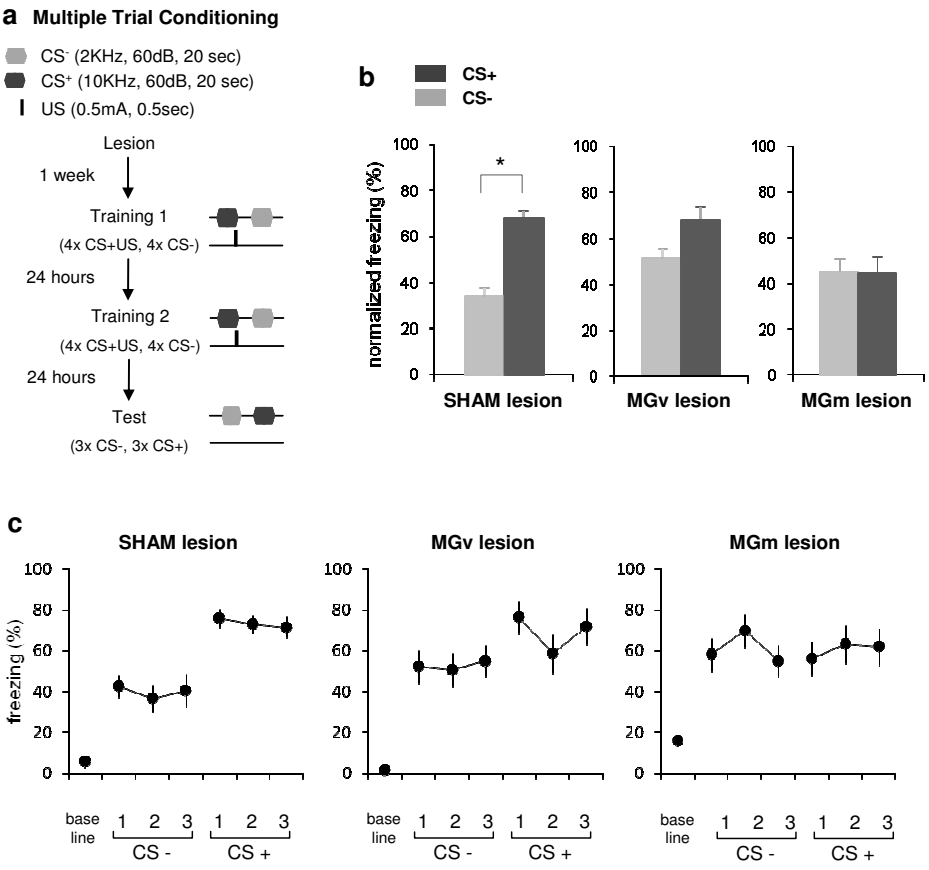

Figure 17 - Both pathways to the amygdala are necessary for the acquisition of discriminative fear. (a) Schematic showing experimental protocol. (b) – (c) Freezing responses to CS+ and CS during discrimination test. Freezing responses are shown as average percent freezing normalized to baseline freezing levels (b) and as raw data, with percent time freezing during the baseline (first Pre-CS presentation), and each presentation of the CS+ and CS- (c). Data presented as mean±sem for n=26 animals (control N=14; MGv-lesion N=7; MGm lesion: N=5). *p<0.0167 (Bonferroni correction).

MGm, but not MGv, is required for the recall of discriminative fear

As expected, in this experiment Sham animals were able to express discriminative freezing between the CS- and the CS+, showing significantly higher levels of freezing to the CS+ than the CS- (Fig. 18b, V=0.0 and p=0.004). Similarly, MGv-lesioned rats showed clear discriminative freezing between the CS+ and the CS- (Fig. 18b, V=2.0 and p=0.0005). In contrast, even though all groups were trained with an intact brain and, thus, acquired normally discriminative fear, MGm-lesioned animals showed impaired expression of discriminative fear, showing similar levels of freezing to the CS+ and CS- (Fig. 18b, V=12.0 and p=0.12). Furthermore, the magnitude of freezing observed in these animals was comparable to that of CS+ evoked freezing in either Sham or MGv-lesioned groups. Hence, generalization between CS+ and CS- in MGm-lesioned animals seems to arise from an inability to suppress freezing to the CS-.

MGm, but not MGv, is required for fear extinction

During the discrimination test, one day after the extinction session consisting of repeated presentations of the CS+ alone, Sham animals showed decreased levels of freezing to the CS+ (CS+ before extinction: 70.0±3.9; CS+ after extinction: 23.3±5.6), which was comparable to freezing to the CS- (Fig. 18c, no difference between CS+ and CS- evoked freezing was observed in the discrimination test after extinction, V=4.0 and p=0.44). MGv-lesioned rats also extinguished freezing to the CS+ (CS+ before extinction: 65.3±3.6;

CS+ after extinction: 40.5±6.6). However, in contrast with control animals, MGv-lesioned rats seemed to display discriminative freezing during the post-extinction discrimination test (Fig. 18c, during the post-extinction discrimination test, a trend for freezing to the CS+ being higher than freezing to the CS- was observed, V=2.0 and p=0.023). As extinction is thought to be a re-learning process, under the conditions of the corresponding experimental protocol, the MGv post-training lesions may in fact be considered as prior to this “new learning”, and the absence of MGv thus causes a “partial extinction” of the CS+ due to generalization between the two tone frequencies. These results are thus consistent with the effect of MGv pre-training lesions on discrimination learning (Fig. 17), because animals were able to extinguish fear to the CS+ but generalized the learned extinction to the CS- so that discrimination between the two stimuli still remained.

Finally, MGm-lesioned rats showed impaired recall of extinction memory of the CS+ (CS+ before extinction: 70.2±6.1; CS+ after extinction: 65.0±7.1). Consistent with the finding that these animals failed to show discriminative fear during the pre-extinction session, in the post-extinction discrimination test they also expressed high levels of freezing to the CS-, comparable to freezing evoked by the CS+ (Fig. 18c, no difference between CS+ and CS- evoked freezing was observed in the discrimination test after extinction, V=2.5 and p=0.112). Moreover, confirming the effect of MGm lesions on fear extinction, a Krukal-Wallis test revealed a significant effect of group on freezing to the CS+ after extinction (K_2=7.5, p=0.024). Posthoc comparisons showed that MGm-, but not MGv-, lesioned animals froze significantly more than control animals to the CS+ after extinction (α=0.0167).

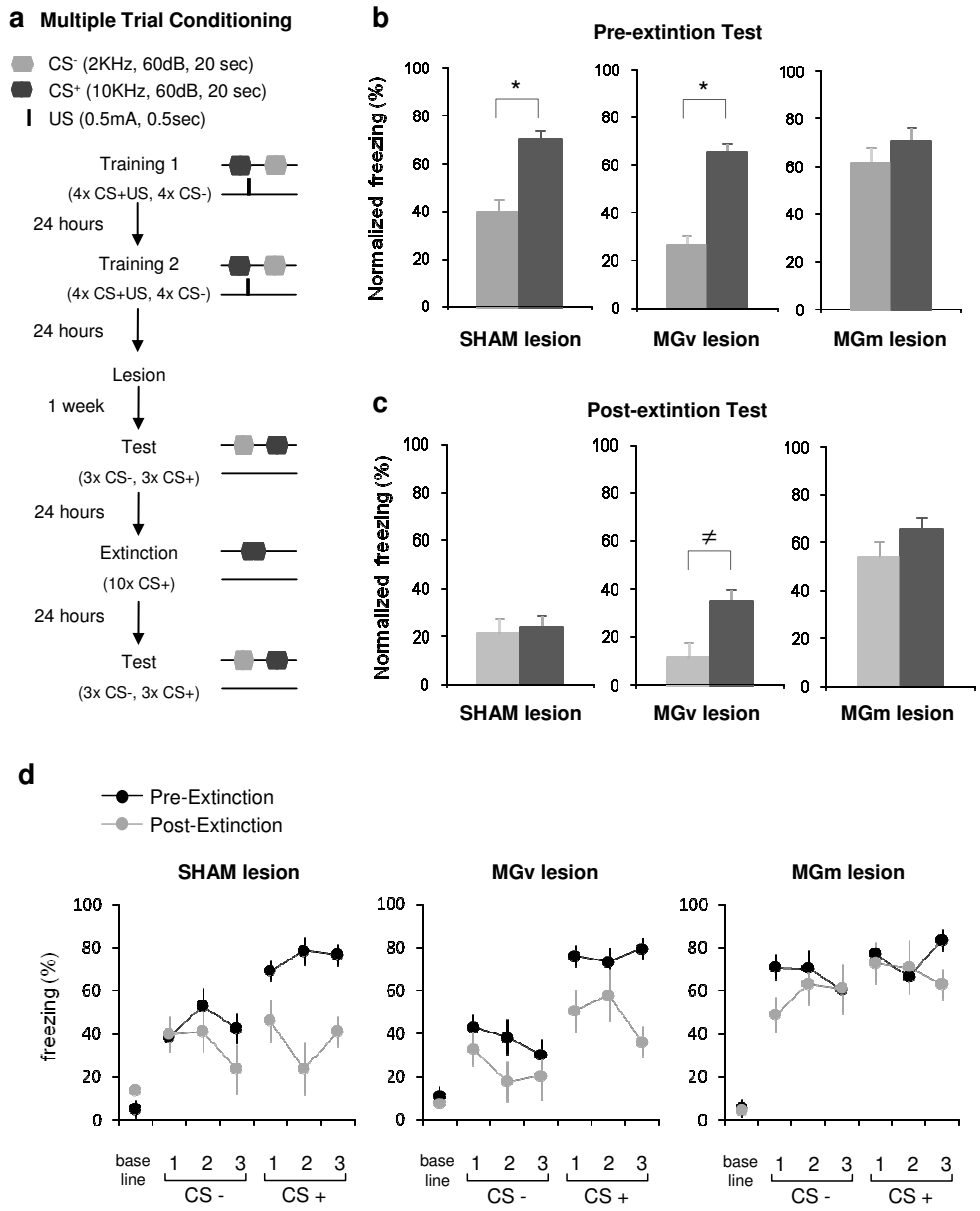
a Multiple Trial Conditioning
CS- (2KHz, 60dB, 20 sec)
CS+ (10KHz, 60dB, 20 sec)
US (0.5mA, 0.5sec)
Training 1
(4x CS+US, 4x CS-)
24 hours
Training 2
(4x CS+US, 4x CS-)
24 hours
Lesion
1 week
Test
(3x CS-, 3x CS+)
24 hours
Extinction
(10x CS+)
24 hours
Test
(3x CS-, 3x CS+)
b
Pre-extintion Test
Normalized freezing (%)
SHAM lesion
MGv lesion
MGm lesion
c
Post-extintion Test
Normalized freezing (%)
SHAM lesion
MGv lesion
MGm lesion
d
Pre-Extinction
Post-Extinction
SHAM lesion
MGv lesion
MGm lesion
freezing (%)
base line
CS -
CS +

Figure 18 – MGm, but not the MGv, is necessary for the expression of discriminative fear and for extinction of fear to the CS+. (a) Schematic showing experimental protocol. (b) - (d) Freezing responses to CS+ and CS- during discrimination test, after lesion (b) and after extinction session (c). Freezing responses are shown as percent freezing normalized to baseline freezing levels (b-c) and as raw data, with percent time freezing during the baseline (first Pre-CS presentation), and each presentation of the CS+ and CS- (d). Data presented as mean±sem for n=29 animals (pre-extinction: control N=8; MGv-lesion N=12; MGm-lesion: N=9; post-extinction: control N=4; MGv-lesion N=7; MGm-lesion: N=5). *$p<0.0167$ (Bonferroni correction); $^{\neq}p<0.05$.

Histology of thalamic electrolytic lesions

Consistent lesions were obtained in both MGv and MGm-lesioned animals (Fig. 19-20). Due to the small size of the MGm, lesions of this nucleus targeted almost its full extent, while MGv-lesions were mainly partial. Nevertheless, even though MGv-lesions were small on a single section (regarding dorso-ventral and medio-lateral extent of the lesions), they spanned through most of the rostro-caudal axis, so that reliable MGv-lesioning could be obtained (Fig. 20). In fact, the great majority of the MGv lesions targeted either the whole extent of each one of the three axes or at least extended, on each one of the axis, from the central region to one of its outermost points (Fig. 21-22).

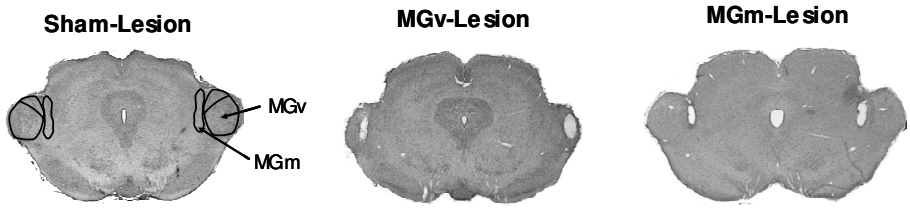

Figure 19 – Coronal sections showing example electrolytic lesions of the thalamic nuclei. MGv: ventral division of medial geniculate body; MGm: medial division of the medial geniculate body; A1: primary auditory cortex; A2: secondary auditory cortex.

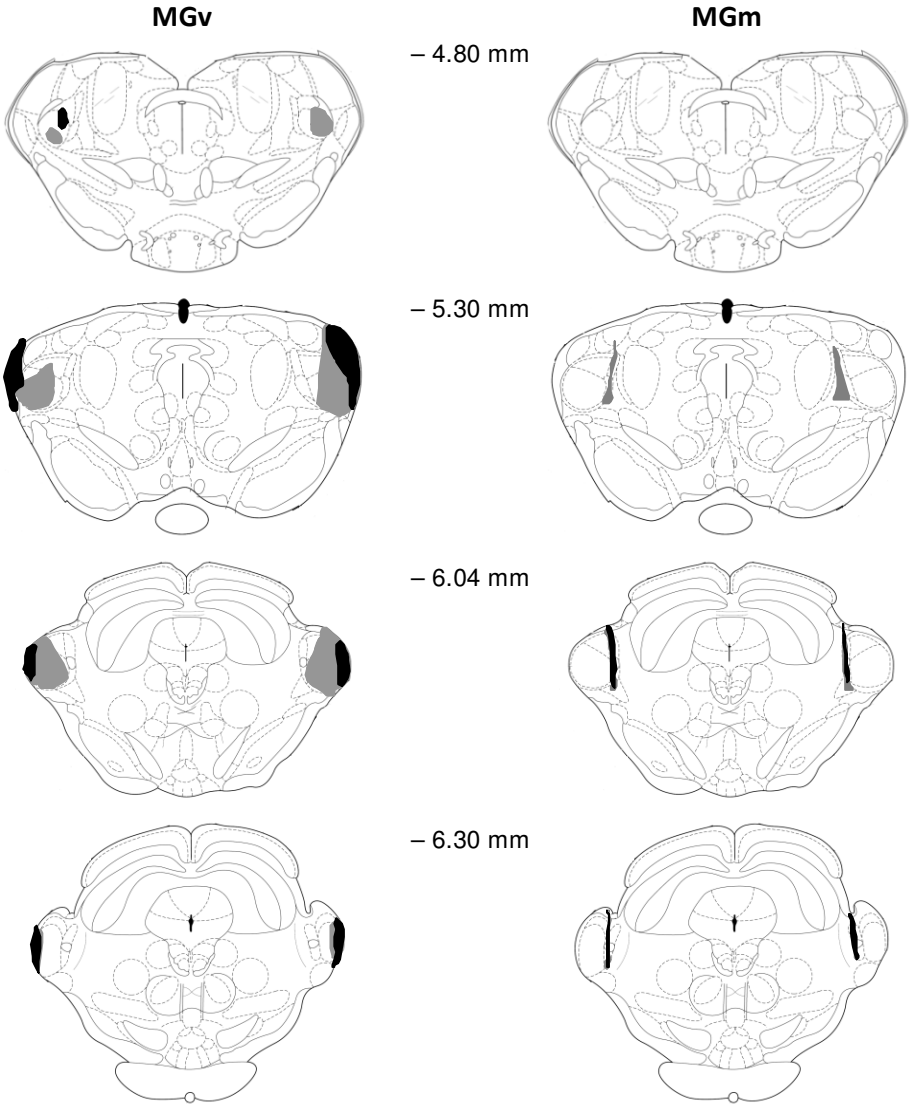

Figure 20 – Schematic representation of bilateral electrolytic lesions of MGv (left) and MGm (right). The largest (gray) and smallest (black) lesions of each nucleus along the anterior–posterior axis (from bregma -4.8 mm to -6.30 mm) are shown in four coronal sections adapted from Paxinos and Watson (1986).

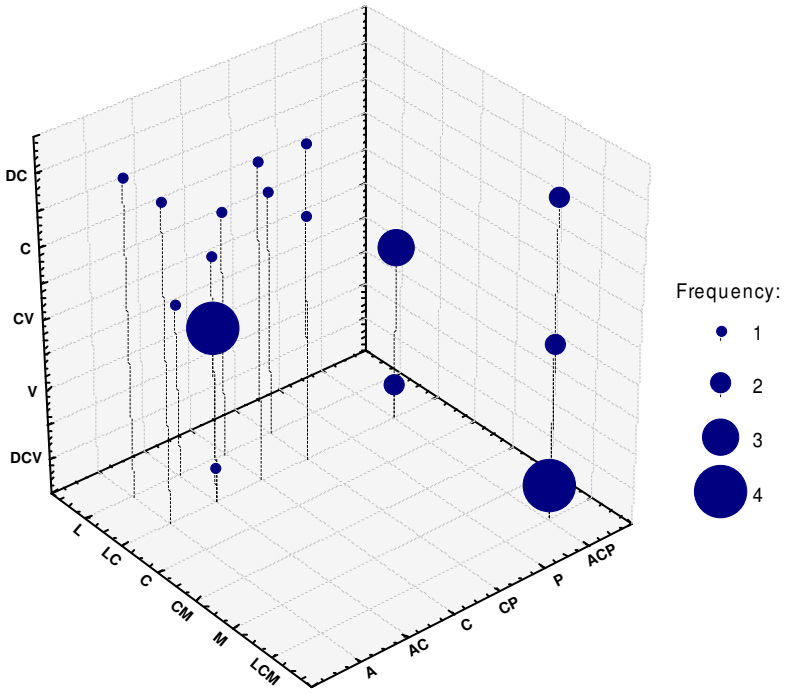

Figure 21 – Extent of the MGv lesions along the antero-posterior, latero-medial and dorso-ventral axis. Graph shows, for MGv-lesioned animals from all experiments, the frequency (absolute value) of lesions placed along the antero-posterior (x), latero-medial (y) and dorso-ventral axis (z). In the antero-posterior axis: ACP for lesions extending along the whole axis; A for anterior lesions; AC for lesions extending in the anterior and central parts; C for central lesions; CP for lesions extending in the central and posterior areas; P for posterior lesions. In the latero-medial axis: LCM for lesions extending along the whole axis; L for lesions placed in the lateral area; LC for lesions encompassing the lateral and central areas; C for lesions in the central area; CM for lesions in the central and medial region; M for lesions in the medial area. In the dorso-ventral axis: DCV for lesions extending along the whole axis; DC for lesions placed in the dorsal and central parts; C for central lesions and CV for lesions extending in the central and ventral areas; V for lesions in the ventral area.

Considering the non-homogeneous lesions of the MGv observed for the whole set of animals (Fig. 21) and the apparently partial results that arose from these lesions (namely in the case of lesions before multiple-trial training, Fig. 22), it is relevant to discuss the topographical organization of the MGv (Bordi and LeDoux, 1994b; Kimura et al., 2003, 2005; Hazama et al., 2004; Winer et al., 1999). This nucleus has very selective neurons organized in a gradient of frequencies which extends along the dorso-ventral axis, with lower frequencies being represented mainly in the dorsal part, and higher frequencies preferentially represented in the ventral part of the MGv (Bordi and LeDoux, 1994b; Winer et al., 1999). Moreover, corticofugal projections from the primary auditory cortex targeting the MGv seem to closely reproduce the dorso-ventral tonotopic map, but further add an organization along the remaining axis that might match gradients of other auditory features (Fig. 9) (Kimura et al., 2003, 2005; Hazama et al., 2004; Winer et al., 1999).

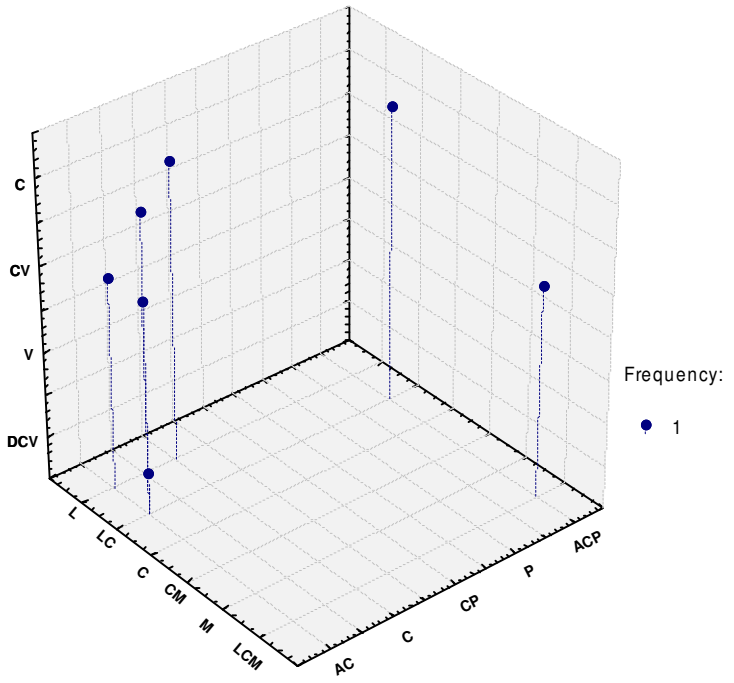

Figure 22 – Extent of the pre-training MGv lesions along the antero-posterior, latero-medial and dorso-ventral axis. Graph shows, for the multiple-trial group of MGv-lesioned animals, the frequency (absolute value) of lesions placed along the antero-posterior (x), latero-medial (y) and dorso-ventral axis (z). In the antero-posterior axis: ACP for lesions extending along the whole axis; A for anterior lesions; AC for lesions extending in the anterior and central parts; C for central lesions; CP for lesions extending in the central and posterior areas; P for posterior lesions. In the latero-medial axis: LCM for lesions extending along the whole axis; L for lesions placed in the lateral area; LC for lesions encompassing the lateral and central areas; C for lesions in the central area; CM for lesions in the central and medial region; M for lesions in the medial area. In the dorso-ventral axis: DCV for lesions extending along the whole axis; DC for lesions placed in the dorsal and central parts; C for central lesions and CV for lesions extending in the central and ventral areas; V for lesions in the ventral area.

We thus further examined the discrimination impairments presently observed according to the histological mapping of MGv-lesions underlying those results. Categorization of the MGv lesions was based on the dorso-ventral axis because it is the one which most consistently supports tonotopic organization.

MGv lesions hypothesized to affect specific frequency ranges were inferred from histological data and classified in one of the following groups: “full range” (lesions extending along the entire dorso-ventral axis, and thus affecting the whole range of frequencies), “middle” (centrally placed lesions, thus affecting middle range frequencies), “middle/high” (lesions on central and ventral areas, thus affecting middle and high frequencies) and “middle/low” (lesions on the central and dorsal areas, thus affecting middle and low frequencies).

It is noteworthy that the present categorization is purely hypothetical, because no recordings have been made to draw the boundaries for each range of frequencies. Nevertheless, neurons in the MGv have been shown to display characteristic frequency (CF, i.e. frequency that evoked responses at the lowest sound level) for frequencies ranging from 1 KHz to 32 KHz (corresponding to the set of frequencies used in a study by Bordi and Ledoux, 1994a). Moreover, hearing in the rat ranges from 500 Hz to ~80 KHz (Sharp, 1998; Paxinos, 2004), and the A1 has been shown to represent frequencies ranging from 1 KHz to about 80 KHz (Rutkowski et al., 2003), namely in the frequency range of ultrasonic vocalizations, above ~20 kHz (Brudzynski, 2005). So, even though the remaining auditory thalamic nuclei seem to preferentially respond to higher frequencies (Bordi and LeDoux, 1994b; Winer et al., 1999), it is plausible to assume that the whole range of frequencies in A1 is also represented in the MGv, and

probably maintained from the cochlea along the lemniscal pathway. Therefore, for the present study, “low frequencies” can be roughly assumed to be those below ~10 KHz (whose representation is even sparse in other thalamic nuclei), and “high frequencies” are those in the upper ultrasonic range, the latter thus not being expected to affect discrimination between the training frequencies.

Using the tonotopic map of MGv to infer affected frequency domains, statistical analysis was then performed on data concerning the pre-training lesions with multiple-trial training, for which an effect of MGv lesions was observed (Fig. 17 and Fig. 23). Comparison between groups was based on differential freezing (*d*, see Methods). In this experiment, three groups of targeted frequencies were observed: “full range”, “middle” and “middle/high”. However, because only one animal had “full range” lesion, it was left out of the analysis, though it curiously had the highest score of differential freezing (*d*=5.14). For the remaining groups a Kruskal-Wallis test revealed no significant effect of group on differential freezing (K_2=4.5, p=0.103, Fig. 23). However, lesions targeting the “middle/high” frequencies appear to impact more on discrimination than “middle” frequencies (Fig. 23), the latter having differential freezing similar to that of sham animals (*d*, Sham: 2.9±0.7; “middle”: 2.1±0.7; “middle/high”: -0.18±1.2). This is probably because lesions affecting “middle/high” frequencies target a wider area, so that a weaker tuned input arises from MGv. Nevertheless, further experiments are required in order to look for a correlation between differential freezing and the size and location of MGv lesions.

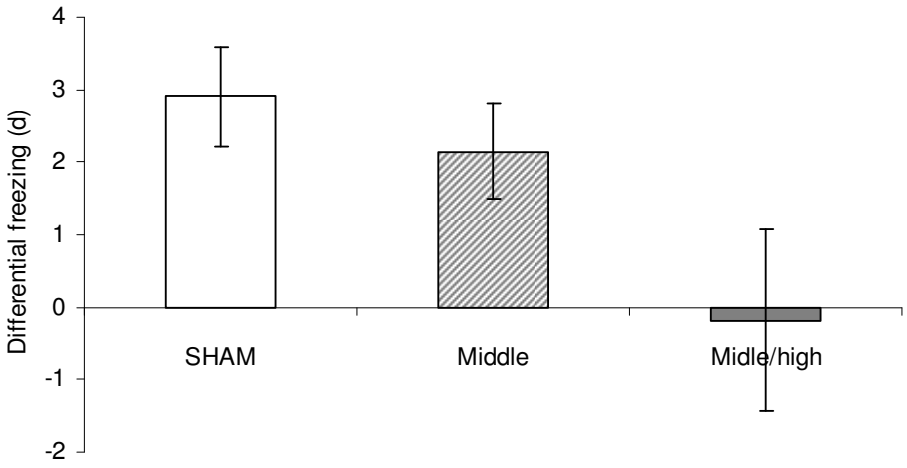

Figure 23 – Effect of MGv lesions targeting different frequency ranges along the dorso-ventral axis on discrimination. Data is presented for the pre-training lesions/multiple- trial training experiment. Graph shows differential freezing (d, see Methods) during discrimination test. Frequency ranges were inferred from histological data, based on MGv tonotopic map (LeDoux et al, 1994a): "full range" - lesions extending along the entire dorso-ventral axis; "middle" – centrally placed lesions; "middle/high" - lesions on central and ventral area. Data presented as mean±sem for N=20 rats (sham N=14, "midle" N=3, "middle/high" N=3). One additional animal having "full range" lesion is not represented. No significant differences between groups were observed.

On the other hand, in the experiments presently reported, because lesions targeting "middle/high" frequencies spare the "low" frequency domain (which theoretically represents the range of frequencies used in this study [CS-, 2 KHz; CS+, 10 KHz]), no

impairment on discrimination would be expected. However, it has been shown that MGv neurons with higher CF generally display narrower bandwidths than neurons with lower CF (Bordi and LeDoux, 1994a), similarly to what is observed in A1 (Kilgard et al., 2001) (Fig. 10). This means that more overlap in neuronal receptive fields probably occurs in areas where neurons have lower CFs, so that lesioning different frequency domains might differently impact on discrimination between tone frequencies. Because the "low" frequency domain has neurons with broader tuning curves, these neurons probably respond to higher frequencies which might lead to generalized responding to the CS-.

Nevertheless, even though frequency is assumed to be the main variable underlying the present experiments, for the overall discussion on the effect of MGv partial lesions, one cannot also discard the possible effects of differently affecting other gradients. In cats, for instance, there is a rostro-caudal gradient of the local GABAergic inhibitory interneurons, the proportion of which increases towards posterior portion (Ehret, 1997). Even though in rats the proportion of inhibitory interneurons is only around 1% (Winer and Larue, 1988), different lesions may differently impact on the MGv results. Therefore, no additional variables can be set aside to explain the present results on MGv lesions, and no absolute considerations can be made regarding those effects because only partial lesions of the MGv were attained.

Discussion

The present study confirms the hypothesis that the indirect lemniscal auditory pathway to the amygdala is necessary for normal discrimination (for a review see LeDoux, 2000), and shows in addition that discrimination also relies on the direct non-lemniscal pathway. Furthermore, we show that even though both pathways are required for intact discriminative learning, their contribution is likely to rely on different mechanisms. The finding that both MGv- and MGm-lesioned rats show intact acquisition of generalized fear responses after single-trial conditioning is consistent with a previous report showing that neither cortical nor MGm lesions affect acquisition of fear of a tone paired with shock (Romanski and LeDoux, 1992). This redundancy in neuronal pathways involved in the acquisition of fear may guarantee self-preservation, even though in single trial learning the learned fear responses generalize to other auditory stimuli.

On the other hand, discriminative fear learning, achieved with multiple trials, requires activity of the two co-existing pathways. The finding that MGv-lesioned rats fail to discriminate after multiple-trial conditioning supports the hypothesis that the indirect lemniscal pathway is crucial for auditory discrimination (LeDoux, 2000). MGv may be important for discriminative learning by facilitating cortical re-tuning (Ma and Suga, 2009), which might enhance the contrast between CS+ and CS- evoked activity in AC, leading in turn to an increase in the CS+ elicited freezing.

It has previously been shown that rats with auditory cortex lesions, when trained to a single pure tone that is paired with footshock, and tested for their fear of tones of different frequencies,

show a generalization gradient similar to that of control unlesioned rats (Armony et al., 1997). This finding was taken as indicative that the auditory cortex was not important for discriminative fear. One possible explanation for the discrepancy between this previous study and our findings is that we trained rats in a discriminative protocol, which may render this task dependent on the indirect cortical pathway, via the MGv nucleus. Moreover, in concordance with the present work, earlier studies have shown that either AC or MGm lesions seem to impair acquisition and expression of differential bradycardia (Jarrell et al., 1986, 1986, 1987; Teich et al., 1988). However, analogies made between conditioned freezing and heart rate changes must be taken cautiously, since these seem to rely on different mechanisms as illustrated by the fact that the acquisition of conditioned heart changes, but not conditioned freezing, to an auditory cue depends on an intact MGm (McCabe et al., 1993).

The finding that MGm lesions also impair discriminative fear learning is at contrast with the “high route/low route” hypothesis, which states that the indirect lemniscal, but not the direct non-lemniscal, pathway is important for auditory discrimination. Our present findings also show that when discrimination is normally learned, expression of the learned discriminative fear responses is impaired by MGm, but not MGv lesions. Thus, although for acquisition of discriminative fear both the lemniscal and non-lemniscal auditory pathways seem to be necessary, the recall of discriminative fear memory seems to rely solely on the latter.

In addition, post-training lesions of the direct non-lemniscal thalamic pathway result in the expression of high freezing levels to both the reinforced and the non-reinforced auditory stimuli, suggesting

that this pathway might be important for discriminative fear by suppressing freezing to the neutral cues. Supporting this hypothesis, Collins and Paré (2000) have shown that in cats there is an increase in unit and field responses to the CS+, and a decrease to the CS- resulting from differential fear conditioning. Consistent with a suppressive role of the direct thalamic input to the amygdala in discriminative fear, we also found that MGm lesions impaired the expression of extinguished fear. This result is consistent with recent findings that the medial geniculate nucleus (including both MGm and MGv) is required for the recall of extinction memory (Orsini and Maren, 2009). Extinction is thought to be a process of re-learning that depends on the inhibition of previously learned responses (for a review see Ehrlich et al., 2009). Therefore, the effect of MGm-lesions on this learning process might result from a disruption of the inhibitory drive onto the amygdala, which is consistent with a significant feedforward inhibition from the thalamus to the amygdala (Pan et al., 2009; Shaban et al., 2006; Shin et al., 2006). This may also be the mechanism by which the MGm is involved in the recall of discriminative fear, which just like extinction, seems to depend on the ability to inhibit freezing, in this case to the CS-. The MGm may play a role in discriminative fear either by suppressing freezing specifically to the CS- or by providing tonic inhibition to the amygdala, working as a gate which only strong inputs like the CS+ could surpass. Supporting this hypothesis, it has been shown that the amygdala is under tight inhibitory control (Amano et al., 2010; Ehrlich et al., 2009; Pan et al., 2009; Shin et al., 2006) and genetically-induced disruption of presynaptic inhibition has been shown to lead to generalization of fear responses (Shaban et al., 2006).

In contrast, post-training lesions of the MGv seem to allow for normal recall of CS+ extinguished memory, even though it generalizes to the non-extinguished CS-, so that discrimination between the two stimuli still remains. Thus the conclusion can be drawn that the MGm on its own is sufficient for extinction learning, but not for discriminatory extinction learning, consistent with the observed impairment in discrimination learning resulting from pre-training lesions. Because extinction is a “new learning”, MGv-lseioned animals were able to extinguish fear to the CS+ but generalized the learned extinction to the CS-, so that discrimination between the two stimuli still remained.

In summary, in the present study we show, on the one hand, that the lemniscal input pathway to the amygdala is necessary for normal acquisition, but not recall, of discriminative fear, possibly by enhancing the representation of relevant relative to the irrelevant cues (Gao and Suga, 2000; Rutkowski and Weinberger, 2005; Weinberger, 2007a, 2007b). On the other hand, our results also suggest that the non-lemniscal pathway is important to suppress fear of neutral or safe auditory stimuli, thereby affecting the acquisition and recall of discriminative fear (as it involves suppression of fear of the CS-) as well as the extinction of fear of an auditory cue that was previously paired with shock. Thus, this work sheds new light into the mechanisms of fear learning and may impact on the understanding of pathological states entailing mal-adaptive fear responses.

CHAPTER IV - Conclusions and Perspectives

The prevalence of defensive responses and fear learning in natural systems, the readiness of how it can be induced and the stability of its memory, and the negative impact on everyday life of maladaptive responses to abnormally processed threat-related stimuli (for a review see Grillon, 2002b) show that fear is a physiological trait with a strong weight on survival and adaptation.

Great progress has been made to understand the mechanisms of fear learning using auditory fear conditioning, and research on the normal fear system may have a major impact in the development of treatment for fear and anxiety disorders (Cryan and Kaupmann, 2005; Cunha et al., 2010). Although the neural circuit underlying auditory fear conditioning is quite well characterized (Fig. 2-3), the present work was aimed to further understand the mechanisms by which an animal can learn to discriminate cues that are predictive of threat, from neutral cues.

The high/low route model (for a review see LeDoux, 2000), which provides a widely accepted mechanism for discriminative fear learning, is based on the electrophysiological properties of the two auditory input pathways into the amygdala. On the one hand, the indirect pathway (high route), via auditory cortex, has neurons that are selective for narrow sound frequency bands, constituting a tuned auditory input (Bordi and LeDoux, 1994b; Kimura et al., 2003; Velenovsky et al., 2003; Storace et al., 2010; Rutkowski et al., 2003). On the other hand, the direct pathway (low route), via the auditory thalamus, has neurons that are not selective, showing a flat response across a wide range of frequencies, thus providing a non-tuned input

to the amygdala (Bordi and LeDoux, 1994b, 1994a; Doron and Ledoux, 1999; Linke et al., 2000). It has thus been proposed that the direct thalamic route to the amygdala is faster but less accurate than the indirect route (LeDoux, 1995, 2000). There was, however, so far, little evidence supporting this model. Furthermore, even though the AC seems to play a critical role in encoding learned importance of sound (Rutkowski and Weinberger, 2005; Weinberger, 2007b), previous studies with pre-training AC lesions or MGm, the auditory thalamic nucleus which projects directly to the amygdala, have shown that each pathway is sufficient to support AFC (Romanski and LeDoux, 1992). However, pre-training lesions may allow neuronal reorganization and the use of different redundant strategies during learning, which does not imply that the non-lesioned brain wouldn't use the auditory cortex as the main pathway for the acquisition of associative fear of the tone CS. Supporting this view, recent studies reported that the same AC lesions, if performed after training, have a strong impact in the recall of the fear of the tone (Boatman and Kim, 2006). According to those authors, post-training AC lesions completely abolished freezing responses to a conditioned tone, with freezing scores almost at 0%. However, the extent of the lesions underlying the reported results, and the nuclei they affected, may all account for a non-specific effect of damaging the perirhinal cortex, which receives multimodal sensory input (for a review see Furtak et al., 2007). Furthermore, whole AC lesions performed by Boatman and Kim were in fact targeting both tuned and non-tuned inputs to the amygdala (via MGm-AC connectivity) on one hemisphere, while completely disrupting auditory pathways on the other, which might explain such low levels of freezing.

Contrasting with the work of Boatman and Kim, preliminary results presented on Chapter II show that the tuned core of the auditory cortex (A1) is not required for the expression of fear memory, thus setting the basis for further studies conducted in context of the current thesis in order to elucidate the role of each pathway of sound to the amygdala in fear learning. Furthermore, these results also show that, in addition to the sufficiency in supporting acquisition of AFC, the direct thalamic pathway is also sufficient for the recall of auditory fear memory. Nevertheless, the existence of parallel streams of information in the auditory fear neuronal circuit and individual contributions of each pathway to amygdala during AFC still need to be clarified.

As the high/low route hypothesis traditionally claims that the cortical pathway is more accurate, though previous work has shown normal discrimination learning in rats with pre-training lesions of AC (Armony et al., 1997), we proposed to test the hypothesis by accessing the role of this pathway in both the acquisition and recall of tone frequency discrimination, thereby performing pre- and post-training lesions. It has been shown that the ascending auditory information on route to the cortex is segregated in two parallel streams, so that the redundancy in the auditory inputs to the amygdala (Fig. 2-3) essentially relies on the structural and functional segregation of inputs in the leminscal (tonotopic) and non-lemniscal (non-tonotopic) systems. Since it is very difficult to assess the borders of A1 histologically, we decided to lesion the inputs to auditory cortex, the auditory thalamus, which has clear histological borders separating the leminscal (tuned) and the non-lemnical (non-tuned) auditory streams. Therefore, further experiments were then designed to access the

contribution of tuned information converging in A1 by selectively disrupting inputs ascending from the auditory thalamus.

By lesioning either the tuned (MGv) or the non-tuned (MGm) thalamic pathways to the amygdala, we have shown that, though each pathway alone is sufficient for the acquisition of generalized fear responses, different roles are played by each pathway in discrimination learning. MGv is necessary for normal acquisition, but not recall, of discriminative fear, and MGm is important to suppress fear of safe auditory stimuli, thereby affecting the acquisition and recall of discriminative fear (as it involves suppression of fear of the CS-) as well as the extinction of fear of an auditory cue that was previously paired with shock.

During discrimination learning, after initial acquisition of generalized fear, an increase in freezing to the CS+ and a decrease in the freezing to the CS- tend to be observed. The sharply tuned MGv is probably involved in discrimination learning by facilitating cortical re-tuning (Yu et al., 2004; Gao and Suga, 2000; Rutkowski and Weinberger, 2005; Weinberger, 2007a, 2007b). But even though this mechanism might account for the increase in the CS+ elicited freezing, on its own it is not sufficient for discrimination as MGm lesions disrupt both the acquisition and expression of discriminative fear, the latter resulting from impaired decrease in responding to the CS- along training.

The present results thus partially support the high/low route hypothesis by showing that discrimination learning requires an intact MGv. In contrast, our results also suggest that the non-lemniscal pathway is important for the acquisition and recall of discriminative fear. The effects of MGm lesions thus raise new intriguing issues in

the study of perceptual discrimination, by showing that a broadly selective structure plays a crucial role in discriminative learning.

To clarify the contribution of the non-lemniscal pathway to discriminative fear learning it would be important to know more about the response properties of amygdala neurons to sound. Although it has been shown that amygdala neurons acquire discriminative responses between a CS+ and CS- (Collins and Paré, 2000), little is known about auditory receptive fields in amygdala, how inhibition and excitation shape the response properties of amygdala neurons and how these receptive fields are shaped by learning. Nonetheless, it has been shown that balanced excitation and inhibition underlie frequency tuning in auditory cortex and that learning leads to changes in both excitatory and inhibitory currents (Wehr and Zador, 2005; Dorrn et al., 2010). Thus, similar mechanisms may operate in amygdala.

Several hypotheses can be put forth to explain the role of the non-lemniscal pathway in discrimination learning. On the one hand, MGm may impact on discrimination learning by the induction or facilitation of plasticity in the cortex, or through convergence with cortical input onto the amygdala neurons. Alternatively, the role of MGm may rely on sustaining inhibitory tone in the amygdala, or in the inhibitory modulation of amygdala neurons, namely through stimulus-specific inhibitory control of interneurons or by interacting with the inhibitory network of the central nucleus. These possibilities are not mutually exclusive and may all contribute to normal learning and expression of discriminative fear.

a) Role of MGm excitatory input onto cortical neurons versus convergence with cortical input directly onto amygdala neurons.

One plausible hypothesis to explain the role of MGm in discrimination is that this nucleus cooperates with the tuned pathway and exerts an effect on frequency discrimination via cortical projections. On the one hand, MGm might provide an excitatory drive that facilitates the output of the selective cortical pathway by directly facilitating plasticity in the cortex via thalamo-cortical projections (Ma and Suga, 2009). However, facilitation of the tuned input to the amygdala via cortex cannot explain the finding that MGm, but not MGv, disrupts the recall or expression of discriminative fear.

On the other hand, MGm also represents a target for corticofugal modulation during associative learning (Kimura et al., 2003, 2005; Shi and Cassell, 1997; Winer and Larue, 1987; Arnault and Roger, 1990; Zhang et al., 2008). Changes in cortical responses to sound could also lead to changes in MGm receptive fields, which may be sharpened and retuned with auditory fear conditioning (Edeline and Weinberger, 1992). This would explain why MGv is important for learning but the expression of discriminative fear would then be mediated by MGm. In any case, the corticofugal modulation loops seem to be important for the improvement and reorganization of subcortical auditory signal processing (Suga et al., 2000; Suga and Ma, 2003; Suga, 2008). Nevertheless, retuning of the MGm alone cannot account for the high levels of freezing observed in the post-training lesions experiments because lesioning MGm would be expected to decrease the excitatory drive to the amygdala, ultimately leading to lower freezing levels.

Finally, MGm input might impact on discrimination through convergence with cortical input onto the same amygdala neurons (Humeau et al., 2005; Shin et al., 2006), thus shaping frequency responding in the amygdala. Supporting this idea, it has been shown *in vivo* that associative LTP can be induced in either the thalamic or cortical input to the amygdala, by stimulating both pathways simultaneously, and that thalamo-amygdala LTP lasts longer than cortico-amygdala LTP (Siguròsson et al., 2010). However, *in vitro* simultaneous activation of converging cortical and thalamic afferents specifically induced associative NMDA-receptor-dependent LTP at cortical, but not thalamic, inputs to the amygdala (Humeau et al., 2003; Shaban et al., 2006). Hence it remains unclear whether associative LTP at amygdala neurons can explain why MGm-, but not MGv-, post-training lesions impair the expression of discriminative fear responses.

Notwithstanding the proposed hypothesis regarding integration of thalamic and cortical inputs, the MGm can itself be a site of CS-US convergence and a learning-induced-source of tuned input to the auditory cortex and the amygdala, further supporting the key role of this nucleus in discrimination learning. Several studies have traditionally identified the amygdala as a critical neuronal substrate for associative learning, pointing the LA as the site where CS-US association takes place. Nevertheless, and opposing to the MGv, single neurons in the MGm/PIN, particularly the PIN, also show convergence of tone and footshock information (Bordi and LeDoux, 1994b, 1994a). Despite its major input being auditory (Linke, 1999a), neurons in MGm/PIN also respond to tactile, thermal, nociceptive, vestibular and visceral stimulation (Bordi and LeDoux, 1994b, 1994a; Weinberger, 2010). In this nucleus, three types of neurons are found:

those responding only to auditory stimuli, those responding to both auditory and somatosensory stimuli, and those responding only to somatosensory stimuli (Bordi and LeDoux, 1994b, 1994a; Weinberger, 2010). Importantly, even unimodal somatosensory cells show increased responses with simultaneous presentation of somatosensory and auditory stimuli (Bordi and LeDoux, 1994b, 1994a; Weinberger, 2010). Furthermore, several data has been pointing these nuclei as more than purely auditory relay stations en route to the amygdala and auditory cortex (for a review see Weinberger, 2010). Accumulating evidence as pointed out the MGm/PIN as an additional site for associative plasticity in AFC (Weinberger, 2010) and as a locus for CS-driven tuning shifts (Edeline and Weinberger, 1992).

The weakly tuned properties of the MGm thus provide a potential window of opportunity for plastic adaptation in the auditory domain, by conveying learning-derived discriminative information to the cortex, amygdala, or both, in an associative-dependent manner. But because MGv lesions also impair discriminative learning, the two thalamic inputs must act in concert to attain discrimination. More importantly, irrespectively of the specificity of the MGm input, an exclusively excitatory contribution from this nucleus cannot account for the observed high levels of freezing resulting from MGm lesions on the expression and extinction of discriminative fear. Complementary or alternative mechanisms must exist to explain the observed results, possibly involving inhibitory modulation of amygdala inputs.

b) Role of MGm on discrimination via inhibitory modulation of the amygdala

Several studies have shown that the amygdala is under tight inhibitory control and that the inhibitory circuits play a role in fear memory acquisition and extinction (Bauer and LeDoux, 2004; Pan et al., 2009; Shaban et al., 2006; Shin et al., 2006; Ehrlich et al., 2009). Furthermore, LTP induction in the LA has been shown to be gated by local inhibitory circuits (Pan et al., 2009; Shaban et al., 2006; Shin et al., 2006; Siguròsson et al., 2010; Ehrlich et al., 2009).

Although the underlying mechanisms are poorly known, it is believed that during fear learning changes in both the excitatory and inhibitory network occur, so that inhibitory tone of the amygdala is maintained (Bauer and LeDoux, 2004; Pan et al., 2009; Shaban et al., 2006; Shin et al., 2006; Ehrlich et al., 2009). Furthermore, accumulating evidence has also been pointing out a role for both excitation and inhibition in modulation of cortical tuning (Wehr and Zador, 2003; Wu et al., 2008; Galindo-Leon et al., 2009; Tan et al., 2004; Sadagopan and Wang, 2010; Dorrn et al., 2010), and it has recently been shown that increased inhibition shapes cortical responding to ultrasonic vocalizations (Galindo-Leon et al., 2009). Because the amygdala is under tight inhibitory control, and despite being broadly tuned (Bordi and LeDoux, 1992; Bordi et al., 1993), it is possible that similar mechanisms of inhibitory plasticity operate in the amygdala nuclei to induce differential firing during discrimination learning.

Synaptic plasticity in cortical and thalamic afferents to the LA is believed to be a mechanism underlying fear learning (Dityatev and

Bolshakov, 2005; LeDoux, 2000) and it has been shown to occur in inputs to both LA principal neurons and interneurons (Szinyei et al., 2007; Bauer and LeDoux, 2004; Pan et al., 2009). Recent studies are unraveling how the interactions between inhibitory and excitatory inputs might shape synaptic transmission and plasticity within the amygdala. Both thalamic and cortical auditory inputs are part of the inhibitory circuit (Bauer and LeDoux, 2004; Pan et al., 2009; Shaban et al., 2006; Shin et al., 2006; Ehrlich et al., 2009), though feedforward inhibition from direct thalamic projections onto inhibitory interneurons appears to be stronger than that coming from the cortex (Bauer and LeDoux, 2004; Pan et al., 2009; Shaban et al., 2006; Shin et al., 2006; Ehrlich et al., 2009) (Fig. 24). Therefore, thalamic projections from MGm may be particularly important in setting the inhibitory tone of the LA. Through silencing of inhibition, LA would become more excitable so that even weaker inputs (safe or neutral signals), which normally wouldn't lead to fear responses, would be sufficient to drive neurons and give rise to fear responses (Fig. 26a). Consistent with this hypothesis, genetically-induced disruption of $GABA_B$-mediated pre-synaptic inhibition has been shown to lead to generalization of fear responses (Bauer and LeDoux, 2004; Pan et al., 2009; Shaban et al., 2006; Shin et al., 2006; Ehrlich et al., 2009), and evidence has been growing on the role of $GABA_B$ receptors in regulating amygdala dependent fear and anxiety (Cryan and Kaupmann, 2005).

Stimulation of LA afferents recruits two main inhibitory mechanisms: feedforward inhibition from interneurons, triggered by thalamic and cortical afferents, and feedback inhibition from interneurons activated by LA principal neurons (Ehrlich et al., 2009) (Fig. 3). And even though there is similar glutamatergic enervation of

principal neurons and interneurons, inhibitory inputs to principal LA neurons are stronger than those to interneurons (Pan et al., 2009), thus setting the basis for plastic inhibitory modulation of LA inputs. Inhibition in the lateral amygdala is modulated post-synaptically at principal neurons via $GABA_A$ receptors ($GABA_AR$) and $GABA_B$ receptors ($GABA_BR$), or pre-synapitcally via $GABA_BR$ at cortical inputs. Pre-synaptic inhibition, resulting from GABA diffusion out of the synaptic cleft, has been shown to be prevalent in principal, but not inhibitory, neurons and to gate plasticity onto excitatory but not inhibitory neurons (Pan et al., 2009) (Fig. 25). Furthermore, LTP of glutamatergic inputs onto inhibitory neurons is not synapse specific (Bauer and LeDoux, 2004; Pan et al., 2009). Thus, taken together, it seems that in LA a number of mechanisms are in place to keep this structure under a plastic inhibitory control, since inhibitory interneurons seem to be less sensitive to GABA and more plastic than principal neurons. And the MGm may thus have an important role in sustaining the inhibitory tone of the amygdala.

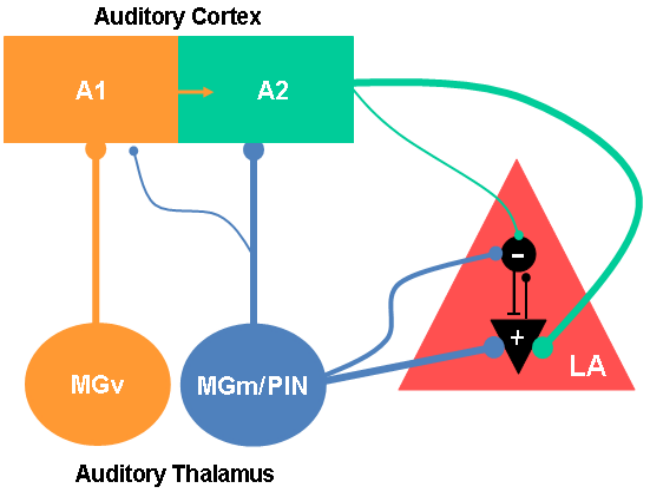

Figure 24 – Cortical and thalamic auditory inputs to the amygdala. Round-ended projections represent excitatory inputs, and dash-ended projections represent inhibitory inputs. MGv: ventral division of the medial geniculate nucleus; MGm: medial division of the medial geniculate nucleus; PIN: posterior intralaminar nucleus; A1: primary auditory cortex; A2: secondary auditory cortex; LA: lateral nucleus of the amygdala; (+): LA principal neurons; (-): inhibitory LA neurons.

On the other hand, though $GABA_BR$-mediated presynaptic inhibition only occurs in glutamatergic inputs due to low accumulation of GABA near the interneurons (Pan et al., 2009) (Fig. 25), it is possible that a stronger excitatory drive onto amygdala neurons *in vivo*, namely during CS-US pairings, leads to higher levels of GABA release which could spill over to interneurons. In this manner, spillover of GABA onto $GABA_BR$ of excitatory neurons would decrease LTP in the cortical inputs to the amygdala, so that only the CS+ elicits firing (Fig. 26a, left panel). Additionally, strong CS+-driven activation of inhibitory neurons might lead to $GABA_BR$-mediated pre-synaptic inhibition of interneurons due to increased local GABA accumulation. $GABA_bR$ activation on interneurons would then lead to a reduction in the inhibitory drive onto the amygdala pyramidal cells in response to CS+, and consequently increase amygdala responses to the CS+, but not to the CS- (which would not drive amygdala neurons sufficiently to increase GABA levels that would spill over to inhibitory interneurons) (Fig. 26b, left panel).

Furthermore, $GABA_c$ receptors, which have recently been proposed to be expressed pre-synaptically on interneurons and act as auto-inhibitors to reduce synaptic GABA release (Cunha et al., 2010), might also play a role in the differential modulation of inhibition in the amygdala.

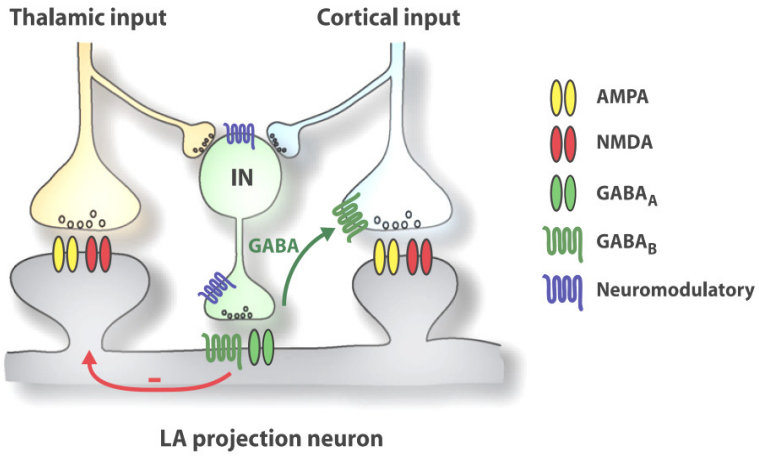

Figure 25 – Inhibitory gating of LTP in the LA (adapted from Ehrlich et al., 2009). Different mechanisms, based on GABA release from interneurons (green), gate the induction of LTP at thalamic (yellow) and cortical (blue) afferents to LA projection neurons (grey).

The thalamic input, representing the major drive for feedforward inhibition (Shin et al., 2006) would thus set the basis for inhibitory gating of the amygdala, by providing sustained inhibitory tone which prevents fear responses to non-fearful stimuli, while silencing inhibition in a stimulus-specific manner during fear learning, thus allowing discriminative responding to CS+ and CS-.

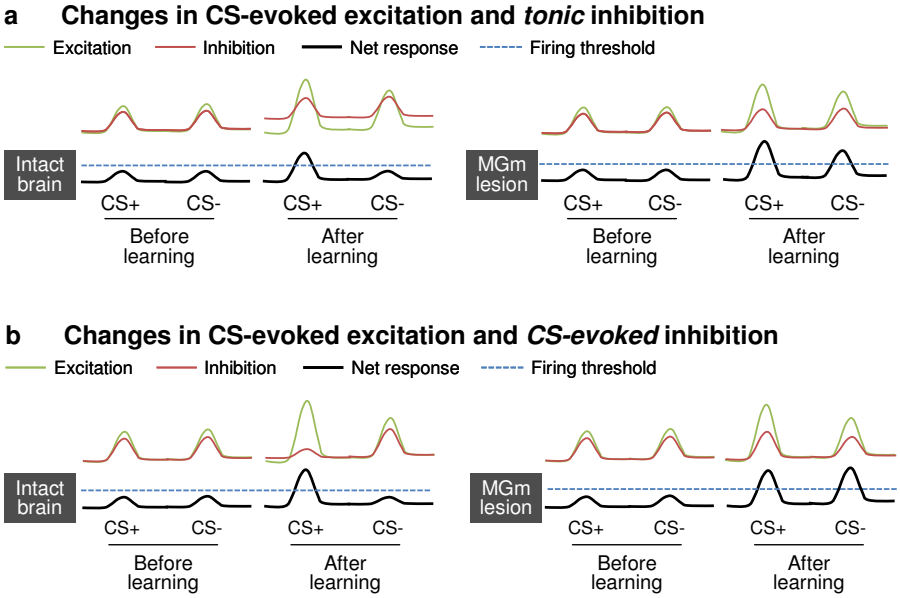

Figure 26 – Proposed mechanisms for the role of the non-lemniscal pathway in discrimination learning. (a) Because feedforward inhibition of LA neurons is stronger from the thalamus, changes in tonic inhibition could mediate discrimination. Taking out MGm might lead to a decrease in the inhibitory tone of the amygdala so that even weaker inputs (CS-) would be sufficient to drive neurons. (b) Spillover of GABA onto $GABA_B$ receptors ($GABA_BR$) of excitatory neurons would decrease LTP on cortical inputs to the amygdala, so that only the CS+ elicits firing, while spillover of GABA onto $GABA_BR$ of inhibitory neurons, for CS+ only, will enhance LTP for the CS+, thus increasing the differences between CS+ and CS-. Taking out MGm could lead to less CS-evoked excitation of amygdala interneurons, thus less spillover of GABA to $GABA_BR$. Therefore, no negative feedback onto excitatory neurons would lead to more LTP even for the CS-, while no spillover to inhibitory neurons would decrease LTP for the CS+, thus leading to impaired discrimination. Both possibilities may contribute to normal learning and expression of discriminative fear. Similar mechanisms can take place in both lateral and central nucleus of the amygdala.

c) Role of CeA in discriminative fear

The central nucleus of the amygdala is another plausible candidate taking part in the inhibitory modulation of the amygdala output, and similar mechanisms to those in the LA may be taking place in CE. This nucleus has been shown to play a role in acquisition and expression of fear responses (McEchron et al., 1995; Wilensky et al., 2006). Lesions of the CE impair the acquisition of fear conditioning (Campeau and Davis, 1995b; Goosens and Maren, 2001; Nader et al., 2001; Ciocchi et al., 2010) as well as the retrieval of fear memory (Ciocchi et al., 2010), and inhibition of protein synthesis impairs the consolidation of fear memory (McEchron et al., 1995; Wilensky et al., 2006). Furthermore, in animals with BLA lesions, conditioned fear responses can be acquired by overtraining in an associative and CE-dependent manner (Rabinak and Maren, 2008; Zimmerman et al., 2007). More importantly, recordings from CE amygdala neurons during fear conditioning have also revealed differential changes in CS+ and CS^--evoked activity in a discriminative fear conditioning paradigm (Pascoe and Kapp, 1985; McEchron et al., 1995), while decreased tonic activity of CE output neurons has been associated with generalization of fear responses to the CS- (Ciocchi et al., 2010).

As in the LA, CE also receives afferent projections from the auditory cortex (McDonald, 1998) and the auditory thalamus (LeDoux et al., 1985b, 1985a; Turner and Herkenham, 1991) (Fig. 3). Projections from the auditory thalamus and cortex, along with projections from the LA, terminate in the lateral division of the central nucleus (CEl) (Linke et al., 2000; Turner and Herkenham, 1991; Pitkänen et al., 1995; McDonald, 1998; Jasmin et al., 1997; Wilensky

et al., 2006). The CEl also serves as the endpoint for nociceptive information (Bernard et al., 1990; Jasmin et al., 1997; McDonald, 1998). In addition, the medial division of the central nucleus (CEm) also receives projections from the auditory thalamus, namely the PIN (Turner and Herkenham, 1991; McDonald, 1998; Linke et al., 2000). Furthermore, converging anatomical and physiological evidence indicates that the CEm, the main output of the amygdaloid complex (LeDoux et al., 1988; Shi and Davis, 1999; Wilensky et al., 2006), is under inhibitory control from the CEl (Sun et al., 1994; Cassell et al., 1999; Ehrlich et al., 2009; Ciocchi et al., 2010).

In what concerns the CE, an increase in CS-evoked firing has been shown in CEm neurons after fear conditioning (Ciocchi et al., 2010). Interestingly, impaired acquisition of fear memory is observed after inactivation of CEl, but not CEm, whereas expression deficits are observed after CEm, but not CEl inactivation (Ciocchi et al., 2010). On the other hand, increased inhibition from intercalated amygdala neurons over the fear output neurons of CEm seems to correlate with fear extinction (Amano et al., 2010). More importantly, it has very recently been shown that plasticity of tonic inhibitory activity within the CEl/CEm circuitry regulates generalization of conditioned fear responses, with an increase of tonic activity of CEl and a decrease in tonic activity of CEm neurons being associated with generalization of behavioral responses to the CS- (Ciocchi et al., 2010).

Altogether these findings support the hypothesis that CE provides an additional site for regulation of fear suppression and expression of auditory discriminative fear responses. Because MGm/PIN neurons are either auditory, somatosensory or multimodal, and even unimodal somatosensory cells show increased responses to

simultaneously presented somatosensory and auditory stimuli (Bordi and LeDoux, 1994a), we put forward the hypothesis that when tone and shock are paired, cells in the MGm/PIN (which show increased firing to the US) might thus send strong inputs to the CE (in addition to LA) due to CS+US association. This thalamic nuclei may then exert its effect over discrimination in diverse ways: by further increasing inhibitory tone of amygdala output neurons (CEm) via direct or indirect cortical projections to CEl, and/or by specifically increasing the activity of CEm neurons in response to US activation through PIN-CEm direct projections (Linke et al., 2000; Turner and Herkenham, 1991).

Despite the inhibitory circuitry is not as well characterized as for the LA, similar mechanisms of inhibitory modulation might occur in the CE and support discrimination (Fig. 26). Whether based on the inhibitory modulation of the CEm by the CEl, or eventually via cortical and thalamic projections onto interneurons like those observed in LA, the CE is also a plausible candidate taking part in discrimination learning via inhibitory modulation. The overall outcome of lemniscal and non-lemniscal projections to the CE would thus be an increased inhibitory tone of the amygdala output neurons via CEl projections, in parallel with a strengthened direct thalamic input to CEm in response to US-predicting tones, thus increasing the signal-to-noise ratio.

This CE modulation may act in concert with the inhibitory modulation of LA interneurons, thus providing a downstream mechanism to further adjust stimulus discrimination. Altogether, these two mechanisms (not mutually exclusive) may possibly underlie the presently reported impairments in discrimination resulting from the disruption of the weakly tuned thalamic pathway to the amygdala.

Based on the hypothesis of inhibitory modulation of the LA and CE, we thus propose that when non-relevant auditory stimuli are displayed (before learning) there is no strong excitatory input to the CE arising from the thalamic or thalamo-cortical-CE inputs. The inhibitory tone of LA and CE is thus maintained by post-synaptic-$GABA_A$-mediated inhibition of amygdala principal neurons and CEl-mediated inhibition of CEm, so that amygdala weakly responds to the neutral sounds and no fear responses are displayed (Fig. 26).

When a biologically relevant stimulus is associated with a previously neutral sound, we propose that the inhibitory tone of amygdala is maintained, so that only stronger stimuli give rise to fear responses (Fig. 26a, left panel). Simultaneously, the CS+ will more strongly drive LA interneurons, so that a higher diffusion of GABA might recruit post-synaptic $GABA_B$ receptors in interneurons, ultimately reducing the inhibitory tone of the amygdala in response to CS+ only, and thus supporting discrimination (Fig. 26b, left panel). Furthermore, a strengthened direct thalamic input arises to CEm in response to US-predicting tones, thus increasing the signal-to-noise ratio and providing for differential freezing to neutral and aversive stimuli.

Discrimination learning without the MGm could thus lead to a decrease in the inhibitory tone of the amygdala, so that even weaker inputs (CS-) would be sufficient to drive neurons (Fig. 26a, right panel), as well as less CS-evoked excitation of amygdala interneurons, thus less spillover of GABA to $GABA_BR$. Therefore, no negative feedback onto excitatory neurons would lead to more LTP even for the CS-, while no spillover to inhibitory neurons would decrease LTP for the CS+, thus leading to impaired discrimination (Fig. 26b, right panel).

Testing the hypothesis

Though several studies seem to at least partially support the hypothesis drawn for presently reported data, further experiments are nevertheless required to test the neuronal mechanisms of auditory discrimination. In particular, because it remains not known whether the MGm is acting via cortical projections or directly via direct projections to the amygdala, a crucial step would be to dissociate the two pathways. To this purpose genetically-induced neuronal inhibition through light-driven chloride pump halorhodopsin (Han and Boyden, 2007; Zhao et al., 2008) or proton pumps (Chow et al., 2010) can be used. Based on the virally-mediated expression of halorhodopsin or proton pumps in the MGm, and by flashing light into the LA, CE or A2, selective silencing of thalamo-amygdala or thalamo-cortical projections can be achieved in the illuminated area, thus clarifying the role of the auditory input from MGm in discrimination learning.

Relevant information may also result from amygdala recordings, by combining a CS+/CS- discrimination protocol with multiple tetrodes for *in vivo* simultaneous recording of neuronal activity in the central and lateral nucleus of the amygdala, as well as MGm. One would thus be able to look for learning-dependent changes in activity of these nuclei, which might underlie differential fear responses.

It would also be valuable to simultaneously record from LA principal neurons and interneurons during discrimination learning, namely using light-activated channelrhodopsin for optical tagging of LA neuronal types during *in vivo* recordings (Lima et al., 2009). To this end, looking for cell-type specific promoters for either inhibitory or

excitatory neurons appears of major relevance (Nathanson et al., 2009; Marik et al., 2010).

On the other hand, *in vivo* whole-cell recordings (Wehr and Zador, 2003) in animals undergoing CS^-/CS^+-training might also provide useful information regarding membrane conductance of inhibitory and pyramidal neurons and receptive field plasticity in the amygdala during learning. Furthermore, combining these approaches with inactivation of the MGv or MGm will further allow looking at the contribution of each sensory input to the modulation of amygdala excitability and frequency tuning during discrimination learning.

Concluding remarks

Overall, the present findings significantly contribute to the understanding of the neural system mediating fear conditioning and discrimination learning, and may thus impact on the study of pathological states entailing mal-adaptive fear responses. More importantly, this work clearly identifies the non-lemniscal auditory thalamus as a key player in modulating discriminative fear learning. However, the role of thalamic nuclei traditionally left aside from these studies, namely the dorsal division of the MGB, which is part of the direct thalamic pathway to the amygdala (Doron and Ledoux, 1999) and which also shows multimodal auditory-somatosensory responses (Ledoux et al., 1987; Bordi and LeDoux, 1994a), might also hide some insights on the poorly known mechanisms of auditory discrimination.

Nevertheless, this work provides data which allowed the creation of testable models to further understand normal fear learning and thus

look for neuronal locus of pathological states of fear, setting the basis for future development of new targeted therapies for pathological conditions. Ultimately, this work brings some insight on how biological systems are hardwired and shaped by life history to adaptively modulate fear responses, and puts forward some hypothesis on the sophisticated tools individuals have been endowed with to fine-tune the expression of fear.

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