Research

Differential dynamics of amino acid release in the amygdala and olfactory cortex during odor fear acquisition as revealed with simultaneous high temporal resolution microdialysis

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Although the amygdala seems to be essential to the formation and storage of fear memories, it might store only some aspects of the aversive event and facilitate the storage of more specific sensory aspects in cortical areas. We addressed the time course of amygdala and cortical activation in the context of odor fear conditioning in rats. Using high temporal resolution (1-min sampling) intracerebral microdialysis, we investigated the dynamics of glutamate and GABA fluctuations simultaneously in basolateral amygdala (BLA) and posterior piriform cortex (pPCx) during the course of the acquisition session, which consisted of six odor (conditioned stimulus)–footshock (unconditioned stimulus) pairings. In BLA, we observed a transient increase in amino acid concentrations following the first odor–shock pairing, after which concentrations returned to baseline levels or slightly below. In pPCx, transient increases were seen after each pairing and were also observed after the last odor–shock pairing, corresponding to the predicted times of anticipated trials. Furthermore, we observed that for the first pairing, the increase in BLA occurred earlier than the increase in pPCx. These data suggest that the amygdala is engaged early during acquisition and precedes the activation of the olfactory cortex, which is maintained until the end of the session. In addition, our data raise the challenging idea that the olfactory cortex might store certain aspects of fear conditioning related to the timing of the associations.

Fear conditioning is one of the most widely used paradigms for studying the neurobiology of emotional learning. This paradigm consists of pairing an initially neutral stimulus (the conditioned stimulus, or CS) with an aversive unconditioned stimulus (US), generally a mild footshock. After a few trials, re-exposure to the CS alone elicits fear responses, such as freezing behavior, which is thought to be part of an anticipatory response to threat and danger (Bevins and Ayres 1995). The major conclusion of these studies is that the basolateral amygdala (BLA) plays a critical role in linking external stimuli to defense responses through synaptic plasticity (for reviews, see Davis 1992; LeDoux 2000; Maren 2001). However, although the amygdala seems to be essential to the formation and storage of fear memories, it might not store all aspects of the aversive event. Indeed, plasticity also occurs in cortical areas during fear conditioning, which could support declarative memories of the learning episode mainly through interactions with the medial temporal lobe memory system (Edeline et al. 1993; McClelland et al. 1995; Quirk et al. 1997; Armony et al. 1998; Weinberger 2004). Thus, the amygdala may store some aspects of fear memory and facilitate the storage of other, more sensory-related, aspects of fear memory in cortical areas (Maren 2005). The precise time course of the differential involvement of the amygdala and sensory cortices in fear conditioning has received little investigation. Until now, most animal studies on this topic have used irreversible lesion or transient inactivation approaches. Very few studies have used approaches with high temporal resolution to investigate changes occurring across dis-

crete trials of a learning session (Quirk et al. 1997; Armony et al. 1998). Furthermore, to our knowledge, no study has compared activation of cortical and amygdalar areas in the same animal during fear conditioning, although this investigation has been done during appetitive learning (Paz et al. 2006).

We chose to investigate the time course of differential activation of regions in the olfactory pathways because, compared with the other sensory modalities, olfactory information has unique direct access to the amygdala, with no obligatory thalamic relay (Price 1973; Savander et al. 1996; McDonald 1998). In turn, the amygdala sends direct projections back to the piriform cortex (Majak et al. 2004). Moreover, the BLA plays a major role in the acquisition, consolidation, and retention of olfactory fear conditioning (Cousens and Otto 1998; Rosenkranz and Grace 2002; Kilpatrick and Cahill 2003; Sevelinges et al. 2004, 2007; Walker et al. 2005; Jones et al. 2007), thus extending previous observations with auditory and visual CSs to include odor cues. Recent studies also suggest that the posterior piriform cortex (pPCx) may play a critical role in this associative learning (Sevelinges et al. 2004, 2008; Jones et al. 2007). Therefore, the olfactory system constitutes a particularly relevant model for studying the relative contribution of sensory cortices and amygdalar nuclei to odor fear learning.

The aim of the present work was to further investigate the time course of activation of amygdala and sensory cortices using *in vivo* microdialysis with high temporal resolution, enabling us to precisely measure dynamic changes in glutamate and GABA levels across successive trials of an acquisition session. Indeed, several pharmacological studies using specific glutamate receptor antagonists have shown that *N*-methyl-D-aspartate (NMDA) receptors in BLA are critically involved in fear acquisition (Miserendino et al. 1990;

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Article is online at <http://www.learnmem.org/cgi/doi/10.1101/lm.1584209>.

Rodrigues et al. 2001; Walker and Davis 2002; Walker et al. 2005), and that NMDA receptor activation is the first step in a cascade of intracellular events, ultimately leading to plasticity that supports learning (for review, see Rodrigues et al. 2004). In addition, local NMDA receptor inhibition in the BLA is thought to prevent learning-induced plasticity, and thereby modify acquisition of conditioned fear (for review, see Ehrlich et al. 2009). However, for both glutamate and GABA, the above-mentioned studies provide only indirect evidence of involvement of the respective neurotransmitters in fear learning. In particular, the occurrence and time course of glutamate and GABA release in vivo during fear acquisition is poorly documented (but see Stork et al. 2002; Young 2004; Venton et al. 2006). In this context, traditional in vivo microdialysis offers temporal resolution in the order of 10–20 min, thus precluding the detection of transient, short-lasting changes. In the present study, using an appropriate analytical method (Parrot et al. 2003), temporal resolution has been lowered to 1 min. This method has enabled us to study rapid changes on a trial-by-trial basis during learning acquisition. Using dual-probe implantation, we compared the time courses of changes in GABA and glutamate concentrations, monitored simultaneously in the BLA and pPCx.

Results

Behavioral study

A retention test was carried out 24 h after the acquisition session to ensure that learning was acquired correctly in the experimental conditions required by microdialysis (i.e., presence of the animals for 3 h in the experimental cage before conditioning, continuous perfusion of the BLA and pPCx).

This retention test was first carried out on three pilot animals of the experimental Odor-Shock group having learned the task under microdialysis. The data presented in Table 1 showed that these animals presented high levels of freezing in response to odor introduction, suggesting that microdialysis during the conditioning session did not alter memory formation or retention.

The retention test was also carried out on a subset of nonoperated animals in the three experimental conditions (Odor-Shock, Shock, Odor), trained as explained in the Materials and Methods, and tested 24 h later for their retention of the learned fear to the odor. Freezing behavior measured in the different experimental groups during the test session is shown in Figure 1. A two-way repeated-measures ANOVA revealed a main effect of group ($F_{(2,15)} = 9.49$, $P < 0.005$) and time ($F_{(7,105)} = 2.37$, $P < 0.05$), as well as a time $\times$ group interaction ($F_{(14,105)} = 4.42$, $P < 0.0001$). Post-hoc tests revealed no statistical difference between groups during the first 2 min of the session prior to CS odor introduction. However, from mins 3 to 8 (i.e., when the peppermint CS was present), Odor-Shock animals exhibited significantly higher levels of freezing than Odor and Shock animals, which were not significantly different from one another. Therefore, in the present experimental conditions, learning was acquired normally as compared with our classical training procedure (Sevelinges et al. 2004).

Table 1. Percentage of freezing (mean $\pm$ SEM) for each minute of the retention test, in pilot animals ($n = 3$) of the experimental Odor-Shock group

Time (min)	1	2	3	4	5	6	7	8
% Freezing	0.00	12.63	97.57	96.70	92.23	85.73	76.03	61.67
$\pm$ SEM	0.00	12.63	1.27	3.30	7.77	14.27	23.97	31.14

Conditioning was done 24 h before under microdialysis in both BLA and pPCx. The CS odor was introduced from min 3 to min 8 as described in the Materials and Methods.

Figure 1. Percentage of freezing (mean $\pm$ SEM) for each minute of the retention test, in the different nonoperated experimental groups. In Odor-Shock ($n = 6$) and Odor ($n = 6$) groups, the CS odor was introduced during the first 20 sec of each minute from min 3 to min 8 (black arrowheads below the x-axis). In the Shock group ($n = 6$), the animal was placed in the experimental cage for 8 min, with no odor stimulation. (*) Significant difference between the Odor-Shock group and the two other groups (Fisher test, $P < 0.05$).

Microdialysis study

In the microdialysis study, animals in the three experimental groups (Odor-Shock, Shock, Odor) were trained as described in the Materials and Methods, while dialysates were collected continuously and simultaneously from the BLA and pPCx at a 1-min sampling rate. Dialysates were collected throughout the duration of the acquisition session, consisting of six trials with a 4-min intertrial interval. Offline glutamate and GABA concentrations present in the dialysates were analyzed (see Materials and Methods) and expressed as a percentage of basal levels measured from the first four samples of the conditioning session. In addition, freezing behavior was quantified throughout the session in the three groups, as described in the Materials and Methods.

Probe placement

Figure 2 illustrates placement of microdialysis probes in the BLA and pPCx in the three experimental groups. Four animals were discarded from the experiment due to inadequate positioning of the probes.

Fear behavior during the conditioning session

Freezing behavior during the conditioning session was quantified for the different experimental groups undergoing microdialysis (Fig. 3A). A two-way repeated-measures ANOVA revealed a main effect of group ($F_{(2,15)} = 48.28$, $P < 0.0001$) and time ($F_{(33,495)} = 14.71$, $P < 0.0001$), and a group $\times$ time interaction ($F_{(66,495)} = 4.26$, $P < 0.0001$). Post-hoc tests showed that the level of freezing of Odor-Shock and Shock animals between mins 5 and 34 was similar and significantly higher than that of Odor animals.

We then compared the freezing scores obtained during acquisition under microdialysis with those obtained during acquisition in nonoperated animals (Fig. 3B). For each experimental condition (Odor-Shock, Shock, Odor), the freezing scores were not statistically different between the nonoperated and the microdialysis animals. In the Odor-Shock group, a two-factors ANOVA showed an effect of time ($F_{(1,9)} = 217.496$, $P < 0.001$), but

Figure 2. Microdialysis probe placements in the basolateral amygdala (BLA) and posterior piriform cortex (pPCx) in the three experimental groups. (A) Odor-Shock group, (B) Odor group, (C) Shock group. Black vertical bars on the histological sections represent the position and length of each microdialysis probe's membrane. Numbers on the right indicate position of the coronal sections, in millimeters, relative to bregma (Paxinos and Watson 1998).

no effect of group ($F_{(1,9)} = 3.273$, $P = 0.104$), as well as no time $\times$ group interaction ($F_{(1,9)} = 0.316$, $P = 0.588$). In the Shock group, the ANOVA revealed an effect of time ($F_{(1,9)} = 44.375$, $P < 0.001$), but no effect of group ($F_{(1,9)} = 0.599$, $P = 0.459$) and no time $\times$ group interaction ($F_{(1,9)} = 3.956$, $P = 0.078$). Finally, in the Odor group, the ANOVA revealed no effect of time ($F_{(1,9)} = 0.827$, $P = 0.387$) and group ($F_{(1,9)} = 3.098$, $P = 0.112$), as well as no time $\times$ group interaction ($F_{(1,9)} = 1.045$, $P = 0.333$).

Glutamate and GABA fluctuations in basolateral amygdala

Glutamate and GABA concentrations were measured from dialysates collected every minute from the BLA and pPCx during

the acquisition session for the different experimental groups. The data obtained from the BLA are presented here.

An ANOVA revealed a main effect of group ($F_{(2,21)} = 3.35$, $P = 0.05$) and a group $\times$ time interaction ($F_{(66,693)} = 1.92$, $P < 0.0001$). There was no significant effect of the neurotransmitter factor. Further pairwise comparisons were then applied among the three groups for each neurotransmitter. For sake of clarity, the data are split into two graphs for each neurotransmitter (Fig. 4). One graph illustrates the comparison between the Odor-Shock and Odor groups, while the other graph illustrates the comparison between the Odor-Shock and Shock groups.

With respect to glutamate fluctuations (Fig. 4, top), post-hoc Fisher tests showed significant differences between glutamate levels measured in Odor-Shock animals and Odor animals (Fig. 4A) during the first trial (min 5) and before the second trial (min 8). This difference was due to an increase in glutamate concentration in Odor-Shock animals at these two time points compared with baseline levels (Wilcoxon comparisons, $P < 0.05$).

Figure 3. (A) Percentage of freezing (mean $\pm$ SEM) for each minute of the conditioning session, for the three experimental groups in microdialysis study. In the Odor-Shock group ($n = 8$), animals received six CS (odor)–US (shock) pairings delivered every 4 min. In the Odor group ($n = 8$), animals received six presentations of the odor alone. In the Shock group ($n = 8$), animals received six presentations of the shock alone. Black arrowheads above the x-axis symbolize the arrival of odor, shock, or odor-shock in the different experimental groups. (*) Significant difference between Odor group and both Odor-Shock and Shock groups (Fisher test, $P < 0.0001$). (B) Comparison of the freezing scores obtained during acquisition under microdialysis (microdialysis groups) with those obtained during acquisition in nonoperated animals (control groups) in the different experimental conditions. Freezing behavior was averaged during two periods of the acquisition session: from minutes 0–4 (before the starting of CS–US pairings) and from minutes 5–34 (during the CS–US pairings). No significant difference was observed between control and microdialysis groups. (*) $P < 0.05$, significant intragroup difference between min 0–4 and min 5–34.

Figure 4. Amino acid concentration fluctuations during the odor fear acquisition session in the basolateral amygdala. Glutamate (top) and GABA (bottom) concentrations were measured simultaneously throughout the session and expressed as a percentage of basal concentration. (A,C) Comparison between Odor-Shock and Odor groups. (B,D) Comparison between Odor-Shock and Shock groups. Black arrowheads above the x-axis in the different graphs symbolize trial occurrence. Light gray vertical bars indicate the timing of the 4-min intertrial intervals. (*) Significant between-groups difference (Fisher test, $P < 0.05$); (#) tendency toward significance (Fisher test, $0.05 < P \leq 0.09$).

No significant differences in glutamate levels were observed after min 8 of the session. Furthermore, the Odor-Shock group had a significantly higher glutamate level than the Shock group (Fig. 4B) for the first trial (min 5), which was due to both an increase in glutamate in Odor-Shock animals and a decrease in Shock animals (Wilcoxon comparisons, $P < 0.05$). In contrast, toward the end of the session, significantly higher levels of glutamate were observed in Shock animals compared with Odor-Shock animals at mins 22, 24, 29, and 30.

With respect to BLA fluctuations (Fig. 4, bottom), we found a significant difference in GABA levels between the Odor-Shock and Odor groups (Fig. 4C) following the second trial (min 10), after which there was a tendency toward slightly higher levels of GABA in the Odor animals, which reached significance at mins 15 and 29. Furthermore, we found a significant difference in GABA levels between the Odor-Shock and Shock groups (Fig. 4D) after the first trial at min 6 (with a tendency toward significance at minute 5, $P = 0.08$). Similar to glutamate, the difference in GABA levels was due to both an increase in GABA in Odor-Shock animals and a decrease in Shock animals (Wilcoxon comparisons, $P < 0.05$). After the second trial, significantly higher levels of GABA were detected in Shock animals at mins 11, 14, 17, 18, 22, 24, 27, and 34, mainly due to a decrease in GABA concentration in Odor-Shock animals (Wilcoxon comparisons, $P < 0.05$).

To summarize our findings in the BLA, we found no global significant difference between changes in glutamate and GABA levels throughout the experiment. Significant transient increases were detected in the Odor-Shock group for both glutamate and GABA levels for the first association, after which a progressive decrease was observed until the end of the session. In the Shock group, a transient decrease in both glutamate and GABA levels was observed for the first association, after which an increase developed slowly as the trials progressed.

Glutamate and GABA fluctuations in the posterior piriform cortex

In parallel with BLA measurements, glutamate and GABA concentrations were measured from dialysates collected each minute from pPCx during the acquisition session for the three experimental groups.

An ANOVA revealed a main effect of group ($F_{(2,19)} = 3.47$, $P = 0.05$), and interactions between group and time ($F_{(66,627)} = 1.43$, $P < 0.05$) as well as time and neurotransmitter ($F_{(33,627)} = 1.46$, $P < 0.05$).

With respect to glutamate fluctuations (Fig. 5, top), post hoc-Fisher tests showed significant differences between glutamate levels measured from pPCx in Odor-Shock animals and Odor

Figure 5. Amino acid concentration fluctuations during odor fear acquisition session in the pPCx. Glutamate (*top*) and GABA (*bottom*) concentrations were measured simultaneously throughout the session and expressed as a percentage of basal concentration. (A, C) Comparison between Odor-Shock and Odor groups. (B, D) Comparison between Odor-Shock and Shock groups. Black arrowheads above the x-axis in the different graphs symbolize trial occurrence. Light gray vertical bars indicate the timing of the 4-min intertrial intervals. (*) Significant between-groups difference (Fisher test, $P < 0.05$); (#) tendency toward significance (Fisher test, $0.05 < P \leq 0.09$).

animals (Fig. 5A) after each of the three first trials (mins 8, 12, and 16) and a tendency toward a significant difference after the fourth trial (min 20, $P = 0.07$). These differences were due to increased glutamate concentrations in Odor-Shock animals compared with baseline levels (Wilcoxon comparisons, $P < 0.05$). In addition, a significant increase was also found at min 29, which corresponds to the predicted time of an additional trial. In comparing Odor-Shock and Shock groups (Fig. 5B), significant differences were observed after each of the two first trials (mins 8 and 11), with a tendency toward a significant difference after the third trial (min 16, $P = 0.09$). These differences were due to both an increase in glutamate concentrations in Odor-Shock animals and a decrease in Shock animals (Wilcoxon comparisons, $P < 0.05$). In addition, significantly higher levels of glutamate were observed in Odor-Shock animals for mins 29–30 and 33–34, which correspond to the predicted times of occurrence of a seventh and eighth trial, respectively.

With respect to GABA fluctuations (Fig. 5, bottom), similarly to glutamate fluctuations, post-hoc Fisher tests showed significant differences in Odor-Shock animals compared with Odor animals (Fig. 5C) after each of the first four trials (mins 7–8, 11, 16, and 20–21). These differences were due to both an increase in GABA concentrations in Odor-Shock animals and a slight progressive decrease in Odor animals (Wilcoxon comparisons, $P < 0.05$). In addition, significantly higher GABA levels were observed in Odor-Shock animals for mins 29–30. In comparing Odor-Shock and

Shock groups (Fig. 5D), significant differences were observed after each of the five first trials (mins 8, 11, 16, 21–22, and 25–26), as well as for mins 29–30 and 34. These differences were due to both an increase in GABA concentrations in Odor-Shock animals and a decrease in Shock animals (Wilcoxon comparisons, $P < 0.05$).

To summarize the findings in pPCx, there was no overall difference between glutamate and GABA fluctuations throughout the experiment. In the Odor-Shock group, significant transient increases were observed following most of the associations. Moreover, additional transient increases were detected after the end of the pairings, at the predicted times at which associations would be anticipated to occur. In the Odor and Shock groups, decreases were observed as the session progressed.

Comparison of the neurochemical dynamics observed in the amygdala and posterior piriform cortex in the Odor-Shock group

In order to better characterize the dynamics of amino acid level fluctuations in pPCx and the BLA in the Odor-Shock group, we compared the glutamate and GABA levels measured in these two structures during the acquisition session.

With respect to dynamic changes in glutamate levels (Fig. 6, top), a two-factor ANOVA revealed a main effect of time ($F_{(33,429)} = 1.70$, $P = 0.01$) and a time $\times$ structure interaction ($F_{(33,429)} = 2.15$,

Figure 6. Comparison of amino acid concentration fluctuations in BLA and pPCx during odor fear acquisition session in the Odor-Shock group. Glutamate (top) and GABA (bottom) concentrations were measured simultaneously throughout the session and expressed as a percentage of basal concentration. Black arrowheads above the x-axis in the different graphs symbolize trial occurrence. Light gray vertical bars indicate the timing of the 4-min intertrial intervals. (*) Significant difference between two structures ($P < 0.05$); (#) tendency toward significance ($0.05 < P \leq 0.09$).

$P < 0.0005$). Post-hoc Fisher tests revealed a significant difference for the first trial at min 5, during which an increase was observed in BLA, while no change was observed yet in pPCx. Beyond the second trial, larger changes in glutamate levels were detected in pPCx than in the BLA for mins 11, 12, 20, and 29.

With respect to changes in GABA levels (Fig. 6, bottom), a two-factor ANOVA revealed a main effect of structure ($F_{(1,13)} = 8.76$, $P = 0.01$) and time ($F_{(33,429)} = 1.62$, $P = 0.02$) as well as a time $\times$ structure interaction ($F_{(33,429)} = 1.71$, $P < 0.01$). Similar to glutamate, the increase detected in BLA in response to the first trial (min 5) preceded the first change in GABA level observed in pPCx, although the difference did not reach significance. Beyond the second trial, larger changes in glutamate levels were detected in pPCx than in the BLA for mins 11, 12, 16–17, 23–25, 29–30, and 34.

In summary, the change in glutamate concentration observed in the BLA preceded the first change in glutamate concentration detected in pPCx. The same tendency was also seen for GABA but did not reach significant levels.

Discussion

The present study provides new insight into the time course of activation of amygdala and olfactory cortex during a fear conditioning acquisition session. High temporal resolution intracerebral

microdialysis allowed us to investigate the dynamics of glutamate and GABA concentration changes in BLA and pPCx with 1-min resolution as learning trials accumulate. To our knowledge, our study is the first to investigate intrasession learning-induced changes in amino acid concentrations, as afforded by the high temporal resolution microdialysis, in both cortex and amygdala in the same animals. We showed that the time course of amino acid level fluctuations in BLA was different from that observed in pPCx. Indeed, odor-shock learning was accompanied by a transient increase in glutamate and GABA concentrations in the BLA following the first odor-shock pairing, after which amino acid concentrations returned to baseline levels or showed a slight progressive decrease. In contrast, transient increases were seen in pPCx after most of the odor-shock pairings and were also observed after the end of the pairings, corresponding to the predicted times of additional trials. Furthermore, we observed that for the first pairing, the increase in BLA occurred earlier than the increase in pPCx.

Since Shock animals exhibited a decrease in amino acid variations in BLA and pPCx while an increase was observed in Odor-Shock animals despite similar levels of freezing in both groups, it can be assumed that the amino acids' increases observed in Odor-Shock animals are not directly linked to shock delivery or to freezing response production, but can rather be ascribed to the learning of the odor-shock association.

Origin of the amino acids collected in the dialysates

Glutamate collected from the BLA and pPCx may have originated from both extrinsic and intrinsic sources. Indeed, the BLA receives inputs from primary olfactory cortex (Haberly and Price 1978; Ottersen 1982; Luskin and Price 1983a) and from more associative areas (McDonald 1998; Sah et al. 2003), providing an extrinsic source of glutamate. Similarly, pPCx receives a strong glutamatergic input from the olfactory bulb as well as inputs from the BLA, prefrontal cortex, and hippocampus (Haberly and Price 1977; Datiche and Cattarelli 1996; Majak et al. 2004). In addition, both the BLA and pPCx contain glutamatergic pyramidal cells that send axon collaterals to neighboring cells, thus providing an intrinsic source of glutamate (Luskin and Price 1983b; Datiche et al. 1996; Sah et al. 2003). Concerning GABA origin, in both BLA and pPCx, GABA is released by local inhibitory interneurons (Sah et al. 2003; Suzuki and Bekkers 2007). The cellular sources of glutamate and GABA measured using microdialysis likely include neurons and glia surrounding the dialysis probe (Watson et al. 2006). Although basal levels of glutamate and GABA measured by microdialysis cannot always be definitively determined, ample evidence exists that increases in neuronal activity lead to increased dialysate concentration of these amino acids (Watson et al. 2006).

In the present study, glutamate and GABA fluctuations were not overall significantly different throughout the experiment, suggesting that GABA increases could be induced by glutamate increases, as shown by Del Arco and Mora (1999) in the prefrontal cortex.

Amino acid fluctuations in basolateral amygdala

In Odor-Shock animals, the first conditioning trial produced a transient increase in glutamate and GABA, while subsequent pairings yielded either no change (for glutamate) or a slight progressive decrease (for GABA). Our data are in agreement with those reported by Venton et al. (2006) in auditory fear conditioning, showing large, rapid, and transient increases in glutamate and GABA levels, but only during the first noise-shock pairing. Similarly, a previous *in vivo* electrophysiological study in rats showed that the greatest fear conditioning-induced changes in single cell responses within the lateral amygdala occurred during the initial acquisition trials, while a decrease in cell responding

was observed during subsequent trials (Quirk et al. 1997). This temporally graded response profile of the amygdala during conditioned fear acquisition is also supported by neuroimaging studies in human showing that the increases of amygdala responses are most pronounced early in fear conditioning, suggesting that the amygdala may be particularly tuned to changes in the relevance of stimuli (Buchel et al. 1998; LaBar et al. 1998; Li et al. 2008).

We postulate that the increase in glutamate concentration observed during the first odor-shock pairing resulted in activation of glutamatergic NMDA receptors, which constitutes the first step of a cascade of cellular events ultimately leading to synaptic plasticity (Lynch 1989; Collingridge and Bliss 1995). Indeed, several pharmacological studies have shown that NMDA receptor blockade in the BLA prior to auditory fear conditioning completely abolished the acquisition of conditional freezing behavior and prevented learning-induced plasticity (Miserendino et al. 1990; Maren et al. 1996; Lee and Kim 1998; Rodrigues et al. 2001, 2004; Bauer et al. 2002; Goosens and Maren 2004; Walker et al. 2005). Therefore, we suggest that glutamate release observed after the first odor-shock pairing in BLA may be responsible for the induction of synaptic plasticity underlying storage of the CS-US association. This hypothesis is in agreement with our previous observation of synaptic facilitation in BLA, as detected during a CS retention test carried out 24 h after odor fear acquisition (Sevelinges et al. 2004). The lack of further increases in glutamate following the next pairings suggests that one pairing is sufficient for the induction of memory trace formation. Behavioral studies have already demonstrated that fear learning can be acquired in a single trial in rodents (Blanchard et al. 1975; Bevins and Ayres 1995; Laurent-Demir and Jaffard 2000). However, it is important to stress that the absence of glutamate peaks from trials 2 to 6 does not mean that no glutamate at all was released during this period. Indeed several studies indicate that fear expression is crucially dependant on AMPA receptors-mediated fast excitatory transmission in the amygdala. For instance, previous studies have shown that infusions of AMPA receptor antagonists into the amygdala interfere with the expression of conditioned fear (Kim et al. 1993; Walker and Davis 1997). Therefore, in our study, small yet physiologically relevant increases might have occurred during trials 2–6 that were not detected by our microdialysis method, but could be involved in the expression of fear observed in our animals until the end of the acquisition session.

In the Shock group, decreased glutamate and GABA concentrations were observed for the first trial, after which a moderate increase slowly developed as trials accumulated. Interestingly, Venton et al. (2006) reported that rats receiving unsignaled shocks during contextual fear conditioning showed no or only weak changes in GABA and glutamate release in the BLA for the initial shock but increased glutamate release during later shocks. Importantly, in the present study, animals in the Shock group did not exhibit contextual fear during the retention test due to context pre-exposure. Therefore, the observed fluctuations in this group cannot be ascribed to contextual learning, but could be ascribed to progressive learning of some temporal aspects of shock presentation (Bevins and Ayres 1995) because we used a fixed 4-min intertrial interval. An interesting point suggested by our data is that even if the Shock animals learned something about the training experience, the dynamics of GABA and glutamate correlated with this learning are quite different from that observed in Odor-Shock animals.

Amino acids variations in pPCx

In Odor-Shock animals, we found that almost every trial was accompanied by a transient increase in amino acid concentrations in pPCx. Because animals in the Odor group showed either no fluctuations (for glutamate) or a slight progressive

decrease (for GABA) it can be assumed that the increases observed in the Odor-Shock animals were not due to the sensory activation alone.

These data further argue for the involvement of pPCx in odor fear conditioning and, more generally, in cognitive mnemonic processes, including learning and recall of associations between odorants and information from other sensory modalities (Litaudon et al. 1997, 2003; Chabaud et al. 1999, 2000; Mouly et al. 2001; Mouly and Gervais 2002; Sevelinges et al. 2004, 2008; Mouly and Di Scala 2006; Jones et al. 2007), while the anterior part of the piriform cortex is more involved in sensory processes and simple forms of memory, such as short-term habituation (Wilson 1998a,b, 2000; Kadohisa and Wilson 2006), pattern completion (Barnes and Thomas 2008), and perceptual learning (Wilson and Stevenson 2003).

Another important finding from the present work is that glutamate increase in pPCx following the first trial occurred ~1–2 min after the increase in the BLA. This observation is in agreement with findings from auditory fear conditioning. Indeed, Quirk et al. (1995, 1997) reported that responses elicited by a tone CS in lateral amygdala cells were modified after pairing with a shock US. Conditioned plasticity is also known to occur in the auditory cortex (Weinberger et al. 1995; Quirk et al. 1997; Weinberger and Bakin 1998). In comparing responses between amygdala and auditory cortex, Quirk et al. (1997) showed that auditory cortex neurons required more trials to learn, and responded more slowly than lateral amygdala neurons within trials. Because our data show that in response to the first trial, the BLA seems to be activated with a slightly shorter latency than pPCx, it may be proposed that the BLA is responsible for the increase in pPCx. This causal relationship may be mediated through the direct pathway between the BLA and pPCx (Majak et al. 2004). The increase in pPCx, in turn, might induce a secondary increase in the BLA, observed just before the second trial, via the pPCx to BLA projection (McDonald 1998). However, we cannot rule out the possibility that another structure receiving afferents from the BLA and sending projections to pPCx might be responsible for the observed effects.

Trial-anticipatory amino acid increases in pPCx

An unexpected finding from the present study was the occurrence of amino acid increases in pPCx in Odor-Shock animals after the end of the associations, at the predicted times of additional trials. This observation seems to indicate that the rats had formed a temporally based expectation of the odor-shock association after only six trials. Evidence for rapid encoding of time interval at the behavioral level in fear conditioning has previously been reported in the literature (Davis et al. 1989; Balsam et al. 2002). In addition, Quirk et al. (1997), recording auditory cortex neurons in freely behaving rats during fear conditioning, reported that cortical cells showed late conditioned responses that seemed to anticipate the US. The investigators suggested that anticipatory firing of cortical neurons may underlie the accurate behavioral timing of CS-US intervals observed during fear conditioning (Davis et al. 1989). The presence of shock-anticipatory conditioned responses in auditory association cortex suggests that sensory areas may contribute to higher cognitive processes, such as timing.

Another interesting finding was the observation of shock-related decreases in amino acid levels in pPCx of Shock animals during the later trials. Interestingly, these fluctuations in pPCx seem to mirror the findings in Odor-Shock animals at the same time points. This could indicate that while pPCx may participate in the timing of odor-shock associations, another structure, such as the BLA as discussed above, or the striatum, which is known to be involved in interval timing (Buhushi and Meck 2005), could

sustain the timing of shocks alone and simultaneously inhibit sensory cortices during shock or in anticipation of its arrival.

Functional interpretations

Although additional experiments are needed to further interpret the present data, we propose that rapid plastic responses in the amygdala may allow fast signaling of novel stimulus–danger associations and drive rapid appropriate behavioral reactions, thus increasing the animal's chances of survival (LeDoux 1996, 2000). Projections from the amygdala to the piriform cortex may provide a pathway through which the amygdala can modulate cortical processing of olfactory information and initiate storage of the various attributes of the learned odor. Thus, the amygdala might store some aspects of fear memory and facilitate the storage of other sensory aspects of fear memory in cortical areas of the brain (Cahill and McGaugh 1998; LeDoux 2000; Maren 2005). This proposal is supported by observations made in human patients with amygdala damage. Indeed, these patients do not exhibit conditioned galvanic skin responses to visual or auditory CSs paired with aversive noises or shock, yet they show normal explicit memory of the circumstances surrounding fear conditioning (Bechara et al. 1995; LaBar et al. 1995). Therefore, in the context of our study, the olfactory cortex might store certain aspects of the conditioning experience, including the learned hedonic value of the CS odor and the accurate timing of CS–US intervals, although this interpretation requires further investigation. In conclusion, due to its anatomical layout, the olfactory system constitutes a particularly relevant model for studying the relative contribution of sensory cortices and amygdalar nuclei to memory processes. The use of a high temporal resolution microdialysis technique has allowed us to reduce the timescale of analysis of the cortico-limbic response to the level of discrete trials during an acquisition session.

Materials and Methods

Subjects and husbandry

A total of 49 male Long–Evans rats (Janvier, France) weighing 250–300 g at the start of the experimentation served as subjects. They were housed individually at 23°C and maintained under a 12 h–12 h light–dark cycle (lights on from 7:00 a.m. to 7:00 p.m.). Food and water were available *ad libitum* during the experiment. Experiments were performed in accordance with the European guidelines regarding the care and use of animals for experimental procedures.

Surgery

The animals were anesthetized with a mixture of ketamine (70 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.) (0.2 mL/100g, i.p.), and were implanted with two stainless steel guide cannulae (CMA/12, CMA) using standard stereotaxic procedures (Paxinos and Watson 1998). The cannulae were implanted in different hemispheres because of surgical constraints. One cannula was implanted in the left BLA (antero-posterior, –2.8 mm relative to bregma; lateral, ±4.9 mm from midline; ventral, –5.5 mm from dura), the other cannula was implanted in the right pPCx (antero-posterior, –1.8 mm relative to bregma; lateral, ±5.5 mm from midline; ventral, –5.7 mm from dura). Therefore, the BLA was always assessed on the left side and pPCx on the right side. The tips of the cannulae were aimed 1.5 mm above the intended areas. The cannulae were fixed to the skull with dental acrylic cement and anchored with a surgical screw placed in the skull. Stylets were inserted into the guide cannulae to prevent clogging. The animals were allowed 2 wk of postsurgical recovery during which they were regularly handled.

Microdialysis probes

Concentric probes were constructed in our laboratory from regenerated cellulose dialysis tubing (Spectra/Por hollow fiber; molecular weight cutoff 6000 Da, 225 μ m O.D., 1.5 mm length,

Spectrum Medical Industries) and fused-silica capillary tubing (90 cm and 120 cm long for inlet and outlet, respectively, 40 μ m i.d., 105 μ m O.D., Polymicro Technology). The body of the probe consisted of a 26-G stainless steel tubing that was glued on a flat probe holder (Harvard) adaptable to a CMA 12 cannula-guide (CMA). An on-line derivatization system was added at the outlet of the probe, as previously described (Parrot et al. 2004). The derivatization reaction took place in the collecting vial. After being flushed with water, the probes were continuously infused with artificial cerebrospinal fluid (aCSF) (145.0 mM NaCl, 2.70 mM KCl, 1.0 mM MgCl₂, 1.20 mM CaCl₂, 0.45 mM NaH₂PO₄, 2.33 mM Na₂HPO₄, pH 7.4). The derivatization reagents were delivered with the same syringe pump as the aCSF (Harvard Model PHD 2000 Infuse) because this pump allowed different flow rates depending on the diameter of the syringe when equipped with a multiple-syringe holder. The aCSF was infused at 2 μ L/min using a 500- μ L syringe. Internal standard (IS) solution (0.1 mM cysteic acid [Cys] in 0.117 M perchloric acid) and naphthalene-2,3-dicarboxaldehyde (NDA) solution (2.925 mM in acetonitrile/water, 50:50, v/v) were delivered at 0.2 μ L/min using 50- μ L syringes. Borate/sodium cyanide (NaCN) solution (mixing solution [100:20, v/v] of 500 mmol/L borate buffer pH 8.7, and 87 mmol/L NaCN in water) was delivered at 0.4 μ L/min using a 100- μ L syringe.

Microdialysis experiments on awake animals require long tubing (~1.5 m) in order to allow the animals to move freely. The dead volume of these tubings (i.e., empty tubing volume between the dialysis membrane and the outlet of the derivatization system) produced dead time that was determined experimentally in order to accurately correlate the neurochemical data with the behavioral events (Parrot et al. 2004). Our probes were specially designed to minimize the dead volume of tubings (1.20 m length, 40 μ m diameter). The resulting dead time of these tubings was 93.3 ± 1.1 sec ($n = 3$). The time course of the microdialysis data was corrected to account for this dead time delay. Moreover, probes with strictly the same geometry (same length of tubings, same length of membranes, same length of probe holders) were used for both the BLA and pPCx, such that a neurochemical event occurring at the same time in both sites would be detected at the same sampling time.

Conditioning cage

The cage consisted of a Plexiglas transparent cylinder (diameter = 21 cm, height = 21.5 cm) with a lateral door, housed in a sound-attenuating enclosure (Sevelinges et al. 2004). The floor of the cage consisted of 17 stainless steel bars, 0.5 cm in diameter, which were spaced 1 cm apart. The floor was connected to a Coulbourn shock generator (Bilaney Consultants GmbH). The ceiling of the cage was perforated with a central aperture (diameter = 2 cm), which allowed the passage of microdialysis tubing when needed. In addition, the ceiling of the cage allowed branching of three Tygon tubings connected to an olfactometer located outside the apparatus. Deodorized air constantly flowed through the cage. At appropriate times, odor was introduced in the air stream. The conditioning cage was placed above a cubic Plexiglas chamber (30 $\times$ 30 $\times$ 15 cm) on which an exhaust fan was mounted, allowing continuous evacuation of the odorant stream from the conditioning cage. The odorant used in the present experiment was peppermint (Sigma Aldrich).

Acquisition session

During the 4 d preceding conditioning, the animals were handled and familiarized to the conditioning cage for 45 min each day. On the day of the experiment, two dialysis probes were implanted into their respective guide-cannulae (BLA and pPCx), and the animal was introduced to the conditioning cage. A 3-h period was used for probe equilibration, after which the conditioning session was initiated and samples were collected at the frequency of one sample per min. Samples were stored at –30°C until analysis for amino acid content.

Three experimental conditions were studied: Odor-Shock, Odor alone, and Shock alone. For the Odor-Shock group ($n = 8$), during the first 4 min of the conditioning session, rats were

allowed free exploration, and then peppermint odor (CS) was introduced to the cage for 20 sec, the last 2 sec of which overlapped with the delivery of 0.4-mA footshock (US). The animal received six pairings of odor and shock, with an intertrial interval of 4 min. After the last pairing, the animal was left in the conditioning cage for 8 min, after which it was returned to the home cage. The total duration of the conditioning session was 34 min. In the Odor group ($n = 8$), the same procedure was carried out except that the odor was presented alone, whereas in the Shock group ($n = 8$), the shock was applied alone. Behavior was videotaped throughout the session for offline analysis.

At the end of the conditioning session, methylene blue (1%) was infused through the probes, the animal was sacrificed with a lethal dose of pentobarbital, and the placement of the probes was verified in the frozen brain (Bert et al. 2004).

Retention of conditioned fear

Because the microdialysis experiment required a 3-h probe equilibration period in the conditioning cage before starting the acquisition session, we wanted to ensure that learning was not altered under these experimental conditions. First, three pilot animals of the Odor-Shock group undergoing microdialysis during acquisition were not sacrificed at the end of the conditioning session and were tested 24 h later for their retention of the task. Second, three additional groups of animals were conditioned using the protocol described above, except that they received no implantation of cannulae. In these animals, conditioned fear was assessed during an 8-min test session carried out 24 h after the acquisition session. During the test session, in the Odor-Shock ($n = 6$) and Odor groups ($n = 6$), the rat was introduced to the experimental cage and no odorant was present during the first 2 min of testing. Peppermint odor (CS odor) was then presented for the first 20 sec of each of the following 6-min epochs. In the Shock group ($n = 6$), animals were placed in the experimental cage for 8 min with no odor stimulation. Behavior during test sessions was videotaped for offline analysis.

Behavior analysis

Behavior was scored offline using an ethological keyboard connected to custom software (Matlab). Behavioral scoring was done by an observer blind to treatment condition. Freezing behavior, characterized by a crouching posture and the absence of any visible movement except that due to breathing (Blanchard and Blanchard 1969), was quantified for each minute of the session. Duration of freezing behavior was expressed as a percentage of the entire minute duration. Freezing percentages were compared using a two-way repeated-measures ANOVA, with group as the between-subjects factor, and time (minute) as the within-subjects factor, followed by post-hoc Fisher tests for pairwise comparisons. For all the statistical comparisons performed, the significance level was set at 0.05.

Microdialysis samples analysis

The samples were analyzed for neurotransmitter amino acid content using an automatic capillary electrophoresis P/ACE MDQ system (Beckman) equipped with a ZETALIF laser-induced fluorescence detector (Picometrics). Excitation was performed using a He-Cd laser (Omnichrome) at a wavelength of 442 nm, the emission wavelength being 490 nm. Separations were carried out on a 63 cm $\times$ 50 μ m i.d. fused-silica capillary (Composite Metal Services) with an effective length of 52 cm. Each day, before the analyses, the capillary was sequentially flushed with 0.25 mol/L NaOH (15 min), ultra-pure water (15 min), and running buffer (75 mmol/L sodium borate, pH 9.20 $\pm$ 0.02, containing 10 mmol/L HP- β -CD and 70 mmol/L SDS) (5 min). The separation conditions were as follows: a 25-kV voltage of 25 kV, 10-sec sample injections at 0.6 psi, cooling temperature between 36°C and 38°C. The capillary was sequentially flushed for 30 sec each with 0.25 mol/L NaOH, ultra-pure water, and running buffer between analyses. Electropherograms were acquired at 15 Hz using P/ACE MDQ software (Sauvinet et al. 2003). The areas of the peaks corresponding to GABA and glutamate were converted to concen-

trations and expressed as a percentage of basal levels measured from the first four samples of the conditioning session. The obtained values were analyzed for statistical significance using a three-way repeated-measures ANOVA, with group and neurotransmitter as the between-subjects factors, and time (minute) as the within-subjects factor, followed by post-hoc Fisher tests for pairwise intergroup comparisons and Wilcoxon tests for intra-group comparisons. For all the statistical comparisons performed, the significance level was set at 0.05.

Acknowledgments

This work was supported by an ANR-07-NEURO-O48 MEMOTIME grant to A.M.M. and Bonus Qualité Recherche from Université Claude Bernard Lyon 1 to R.G. We thank Belkacem Messaoudi for technical assistance with this project.

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Received August 7, 2009; accepted in revised form September 11, 2009.

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Learn. Mem. 2009, **16**:

Access the most recent version at doi:[10.1101/lm.1584209](https://doi.org/10.1101/lm.1584209)

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