Research

Post-acquisition release of glutamate and norepinephrine in the amygdala is involved in taste-aversion memory consolidation

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Amygdala activity mediates the acquisition and consolidation of emotional experiences; we have recently shown that post-acquisition reactivation of this structure is necessary for the long-term storage of conditioned taste aversion (CTA). However, the specific neurotransmitters involved in such reactivation are not known. The aim of the present study was to investigate extracellular changes of glutamate, norepinephrine, and dopamine within the rat amygdala using in vivo microdialysis during the acquisition and 1-h post-acquisition of CTA paradigm. Microdialysis monitoring showed a significant norepinephrine increase related to novel taste exposure and a glutamate increase after gastric malaise induction by i.p. LiCl administration. Interestingly, we found a spontaneous concomitant increase of glutamate and norepinephrine, but not dopamine, 45 min after conditioning, suggesting the presence of aversive learning-dependent post-acquisition signals in the amygdala. These signals seem to be involved in CTA consolidation process, since post-trial blockade of N-methyl-D-aspartate or β-adrenergic receptors impaired long- but not short-term memory. These data suggest that CTA long-term storage involves post-acquisition release of glutamate and norepinephrine in the amygdala.

The amygdala has been established as an important locus for the processing of aversive signals and their association with a variety of cues (McGaugh et al. 2002; McGaugh 2004; Pape and Pare 2010). It has been shown that when animals associate a taste with digestive malaise, a reliable aversion for that particular taste is induced, called conditioned taste aversion (CTA). Thus, the exposure to a novel taste like saccharin solution induces neural changes in the basolateral (BLA) and central (CeA) nuclei, (Yamamoto et al. 1997), and the gastric malaise induction is related to an increase of extracellular glutamate in the BLA (Tucci et al. 1998; Miranda et al. 2002). The association between novel taste and gastric malaise is efficiently consolidated, since only one trial is required to form a long-lasting aversive taste memory.

It has been proposed that long-term stabilization of memory traces involves post-learning neuronal reactivation in the absence of sensory input. These processes may need reactivation of the biochemical pathways triggered by neurotransmitters release to sustain levels of protein that are required for persistence of memory. Recently, we demonstrated that the insular cortex (IC) becomes reactivated in the post-acquisition stage through glutamate and dopamine release; particularly, the reactivation of N-methyl-D-aspartate receptors (NMDAr) is involved in the consolidation process. Such reactivation is dependent on the amygdala functional integrity, since the temporal blockade of this structure with tetrodotoxin before the neurochemical reactivation impairs both dopamine and glutamate release in the IC and CTA consolidation (Guzmán-Ramos et al. 2010).

There is evidence that catecholamines have strong modulatory influences in the consolidation process within post-learning periods (LaLumiere et al. 2004; LaLumiere and McGaugh 2005). It has been reported that post-trial infusions of antagonist or agonists of β-adrenergic receptors, impaired or enhanced, respectively, inhibitory avoidance consolidation (Gallagher et al. 1977; Ferry and McGaugh 1999). In the CTA paradigm, norepinephrine within the amygdala seems to play a critical role during the acquisition stage (Miranda et al. 2003), but there is scarce information about the modulatory effects on post-acquisition stages in order to consolidate the taste memory trace.

Therefore, the purpose of the present study was to evaluate by in vivo microdialysis the release of glutamate, norepinephrine, and dopamine during the presentation of taste and inducing malaise agents, and during a 60-min period after the association phase has occurred. In addition, we tested whether such post-acquisition events would have a functional role in long-term memory stabilization.

Results

In order to evaluate glutamate, norepinephrine, and dopamine release in the amygdala, the following groups of animals were submitted to in vivo microdialysis monitoring during stimuli exposure and one extra hour. The conditioned group (SAC-LiCl n = 6), was exposed for 15 min to 10 mL of a 0.1% (wt/vol) sodium saccharin solution, followed by an i.p. LiCl injection (0.4 M, 7.5 mL/kg) 15 min later. Rats in the nonconditioned group (SAC-NaCl, n = 6) were exposed to the same saccharin solution, but afterward they received an i.p. NaCl injection (0.4 M, 7.5 mL/kg) that does not cause gastric malaise and, therefore, no taste aversion developed. Another control group of animals was exposed to tap water instead of saccharin and received the LiCl injection after 15 min to rule out the possible effects of LiCl by itself (H2O-LiCl, n = 7). Finally, the backward conditioned group (LiCl-SAC, n = 6) was exposed to a LiCl injection (0.4 M, 7.5 mL/kg) and 15 min later the saccharin solution was presented, resulting in an ineffective training for CTA memory formation (Barker and Smith 1974; Guzmán-Ramos et al. 2010).
Release of glutamate during CTA training and post-acquisition period

The baseline concentration of glutamate was 2.34 ± 0.23 pmol/μL in the microdialyzed groups. Two-way ANOVA analysis of glutamate monitoring indicated a significant difference among groups (SAC-LiCl group and its control groups SAC-NaCl, H2O-LiCl, and LiCl-SAC). F(2,3307) = 5.604, P < 0.01 and among fractions (F(20,979) = 3.695, P < 0.01). Figure 1A shows no changes in glutamate release in the amygdala during the saccharin presentation in both the SAC-LiCl (conditioned group) and the SAC-NaCl group. Analysis of the glutamatergic changes in the SAC-LiCl group showed differences among fractions, particularly the administration of LiCl 0.4 M induced a significant increase in glutamate release in the 45-min fraction (F(20,97) = 3.228, P < 0.01), while in the SAC-NaCl group the administration of an equimolar solution of NaCl was not able to induce it (F(3,20) = 2.923, P < 0.05, SAC-LiCl vs. SAC-NaCl in the 45-min fraction). Interestingly, post-hoc analysis in the 85-min fraction during post-acquisition monitoring revealed a significant increase in glutamate in the SAC-LiCl group, but not in the SAC-NaCl or in other control groups (F(1,18) = 4.051, P < 0.05). To rule out that the LiCl administration alone is involved in the post-acquisition increment, we compared the glutamate release of the conditioned group with another control group that tasted tap water instead of saccharin before the LiCl i.p. injection (H2O-LiCl). As we can see in Figure 1B, glutamate levels remained unaffected by the water intake, and the subsequent LiCl administration induced glutamate increases ~250% of baseline release (F(20,107) = 2.608, P < 0.01 in fraction 45 min). However, no post-acquisition changes were seen in this group, indicating that LiCl administration alone, without previous SAC exposure, did not induce post-acquisition glutamate increase. We also evaluated and monitored a group that underwent backward conditioning; in this protocol the CS is preceded by the US presentation and CTA cannot be established (see Figs. 1C, 2). In this group, the LiCl administration also induced a significant glutamate release (F(20,93) = 2.897, P < 0.01 in fraction 20 min) and the saccharin solution had no effect on extracellular glutamate. Nevertheless, the post-acquisition glutamate increments were not present, proving that only the CS–US forward conditions induce glutamate post-learning activity.

Release of norepinephrine during CTA training and post-acquisition period

The baseline concentration of norepinephrine was 5.23 ± 0.53 fmol/μL. Statistical analysis made by two-way ANOVA revealed significant differences among groups (F(3,382) = 6.172, P < 0.01) and among fractions (F(20,382) = 6.460, P < 0.01). During the monitoring of CTA acquisition (Fig. 1D), a significant increment of ~300% of baseline was found in the 25-min fraction during exposure to the saccharin solution (F(20,98) = 2.107, P < 0.01) in the SAC-LiCl group. In the SAC-NaCl group, significant changes in norepinephrine related to the saccharin exposure were also found (F(20,100) = 4.083, P < 0.01). In both groups the LiCl and NaCl administration (fraction 45 min) induced transient and similar increment of extracellular norepinephrine (SAC-LiCl group F(20,98) = 2.107, P < 0.01 and in SAC-NaCl group F(20,100) = 4.083, P < 0.01). However, the continuous post-acquisition monitoring revealed a 210% increase exclusively in the SAC-LiCl at 45 min after LiCl injection (85-min fraction, F(3,17) = 2.527, P < 0.05). Therefore, this increment was significantly different from the SAC-NaCl and the other control groups that did not develop taste aversion (see Fig. 2). It is noteworthy that even though both SAC-LiCl and SAC-NaCl groups showed the same noradrenergic increments related to the CS–US presentation, only the SAC-LiCl group elicited the post-acquisition release. Significant changes of norepinephrine were found in the H2O-LiCl control group (Fig. 1E), (F(20,102) = 3.709, P < 0.01). The water presentation did not elicit norepinephrine release, unlike a novel taste exposure such as saccharin that did produce a significant norepinephrine release (Fig. 1D); thus, the only noradrenergic change found in this group was related to LiCl administration (F(20,102) = 3.709, P < 0.01 in fraction 45 min). Although the backward conditioned group showed significant norepinephrine changes (F(20,90) = 2.174, P < 0.01) with the administration of LiCl (F(20,90) = 2.174, P < 0.01 in fraction 20 min compared with baseline fractions) and saccharin solution presentation (F(20,90) = 2.174, P < 0.05 in fraction 45 min), the post-acquisition activity was absent (Fig. 1F). This finding is in agreement with the fact that the groups that did not acquire CTA learning did not show the neurochemical reactivation.

Release of dopamine during CTA training and post-acquisition period

Basal concentration of dopamine was 6.35 ± 0.13 fmol/μL. Although overall analysis showed no significant differences among groups (F(3,394) = 2.213, P = 0.086) and among fractions (F(20,394) = 1.146, P = 0.29). As it can be seen (Fig. 1G–I), no significant post-acquisition increments of dopamine were present in any behavioral condition, suggesting that this neurotransmitter may not be involved in CTA consolidation in terms of neurochemical reactivation in the amygdala.

Behavioral results

Three days after training, aversion memory was evaluated by re-exposing the animals to the saccharin solution, and their consumption was measured.

As we have seen in Figure 2, the behavioral results showed that the conditioned SAC-LiCl group produced a significant and clear taste aversion. On the contrary, the H2O-LiCl, SAC-NaCl, and the backward conditioning (LiCl-SAC) control groups failed to elicit reliable CTA. The ANOVA test showed significant differences among groups, (F(2,49) = 34.1101, P < 0.0001) and a post hoc analysis indicated that the saccharin consumption in the LTM test of the SAC-LiCl group was significantly different from the rest of the control groups (P’s < 0.0001).

Post-trial blockade of NMDA and β-adrenergic receptors impairs CTA consolidation

Our microdialysis results indicate that there is post-acquisition activity of glutamate and norepinephrine in the amygdala after CS–US association. Therefore, we evaluated the functional role of such post-acquisition neurochemical changes by blocking either the NMDA glutamate receptors (APV, 10 μg/μL) or the β-adrenergic receptors (propranolol, 5 μg/μL) 30 min after the acquisition period. A simple ANOVA analysis showed no significant difference among groups in the STM test, indicating that drug administration did not impair the task acquisition (Fig. 3). However, significant differences were found among groups in the LTM test, as revealed by one-way ANOVA analysis (F(2,49) = 16.563, P < 0.0001). Post-hoc Fisher test showed that LTM was impaired in the NMDA-inhibited group (APV), as it was significantly different from the saline group (SS), which developed a clear long-term aversion (P < 0.01). The group that received intra-amygdalar propranolol (PROP) also showed differences in the LTM test compared with the SS group (P = 0.0015). However, the LTM consumption of the PROP group was significantly different from the APV group (P = 0.0071), indicating that although post-acquisition activity of both NMDA and β-adrenergic receptors
is involved in the CTA consolidation process, the NMDAr blockade caused a larger impairment, leading to the consumption of almost 100% of the saccharin consumed during acquisition.

Discussion
The behavioral results showed that the only group having clear taste-aversion response is the one that received the forward
association between saccharin and LiCl. In comparison, the groups that received saccharin-NaCl or H2O-LiCl presentations or LiCl-saccharin backward association did not show taste aversions. The microdialysis results showed that the presentation of a new taste induced increase in norepinephrine, but not in dopamine release. The intraperitoneal administration of LiCl induced increased release of glutamate, which was specific for the induction of gastric malaise, since an equimolar solution of NaCl did not post the post-acquisition monitoring showed a concomitant increase in norepinephrine and glutamate ~45 min after the association of stimuli (saccharin-LiCl). Interestingly, these changes were observed only in the group that received the forward association, while the aforementioned control groups (saccharin-NaCl, H2O-LiCl, and LiCl-saccharin) did not show the post-acquisition activity of glutamate and norepinephrine. Finally, pharmacology results indicated that the blockade of either NMDA or β-adrenergic receptors in the amygdala 30 min post-acquisition impaired LTM but not STM, indicating a specific role of these receptors in CTA consolidation.

After CTA acquisition takes place, several cellular mechanisms are engaged to carry on the consolidation process. We have proposed that among the required signals for this process are post-acquisition neurochemical reactivations of the structures that were initially involved in the acquisition stage, such as the IC and the amygdala (Guzmán-Ramos et al. 2010). In this regard, we showed that the amygdala is engaged during acquisition through noradrenergic and glutamatergic responses related to the CS and US presentation, and then it becomes reactivated ~45 min later with significant release of glutamate and norepinephrine. The amygdala activity during saccharin presentation might be related to the novelty of the stimulus; several studies have reported increased amygdalar electrical activity and c-fos expression related to the exposure of new gustatory stimuli (Yamamoto et al. 1997; Nishijo et al. 2000; Fontanini et al. 2009). In terms of neurotransmitter extracellular changes, food consumption is associated with increase of acetylcholine, dopamine, and norepinephrine in the amygdala (Savard et al. 1983; Hajnal and Lenard 1997; Hajnal et al. 1998). In the present study we only found significant changes of norepinephrine, but not dopamine. In contrast, Hajnal and Lenard (1997) found that the dopamine release was associated with food consumption related to the increase of glucose and insulin levels, rather than to the novelty of the food, since the animals were tested with the same food of which they were deprived. In the present study, we found significant increases of norepinephrine with saccharin, but not with water presentation, indicating that the novelty of the gustatory stimulus, and not just the liquid consumption, could be causing the noradrenergic response. In this regard, it has been demonstrated that intramygdalar injection of norepinephrine before taste exposure increases the preference for the familiar tastes over novel gustatory stimuli, i.e., increasing the neophobic response (Borsini and Rolls 1984). Novel stimuli represent relevant cues for aversive or appetitive outcomes, and amygdalar activity may modulate the strength of the association stimulus-consequence through the noradrenergic system. Our results also indicate that novel taste stimulus does not produce extracellular changes in glutamate within the amygdala, which is in accordance with previous results (Tucci et al. 1998; Miranda et al. 2002). In summary, amygdala norepinephrine but not glutamate or dopamine release seems to be related with the relevance or salience of the novel stimulus presentation.

The amygdala activity during the gastric malaise induction has been well documented (Yamamoto et al. 1997; Miranda et al. 2002; Bermudez Rattoni 2004; Bernstein and Koh 2007); particularly, glutamatergic activity seems to be important for the aversive signal that is associated with the previous gustatory stimulus (Tucci et al. 1998; Yasoshima et al. 2000; Miranda et al. 2002). In the present study, we confirmed that the glutamatergic increments are specific to the induction of gastric malaise, since the administration of an equimolar concentration of NaCl did not elicit changes in the amygdalar extracellular glutamate and failed to produce CTA memory. On the other hand, the noradrenergic signal turned out to be less specific, since both NaCl and LiCl solutions induced similar norepinephrine increments in the amygdala. The norepinephrine increase during administration of the NaCl solution may be part of a stress response caused by the administration of irritating solutions in the peritoneal cavity, since both NaCl and LiCl solutions were hypertonic, which

Figure 2. The conditioned group is the only one that shows aversion in a long-term memory test. The microdialyzed groups were exposed to the saccharin solution 72 h after stimuli exposure. For a description of the groups, see Figure 1 legend. The SAC-LiCl conditioned group showed a reliable taste aversion. However, the control groups—SAC-NaCl, H2O-LiCl, LiCl-SAC—did not show CTA. Graphics are expressed as % of consumption of saccharin during acquisition (milliliter of saccharin during test × 100)/ml saccharin during acquisition) ± SEM. (** P < 0.01 vs. SAC-LiCl.

Figure 3. NMDA and β-adrenergic receptors activity during post-acquisition is required for memory consolidation. (A) Effect of intramygdalar administration of APV and Propranolol 30 min after CTA acquisition in short- and long-term memory. Post-acquisition blockade of NMDAr (APV group) or β-adrenergic receptors (PROP group) impaired CTA consolidation compared with the group that received the saline injection (SS). (B) Experimental protocol during CTA acquisition, drugs administration (white triangle), and memory tests. The results are showed in consumption of saccharin during acquisition (milliliter of saccharin during test × 100)/ml saccharin during acquisition) ± SEM. (STM) short-term memory; (LTM) long-term memory; (** P < 0.01 vs. SS group; (6) P < 0.01 PROP vs. APV group.
originate a nociceptive signal that activates the LC neurons (Robbins and Everitt 1995), hence inducing an elevation of noradrenaline release in the amygdala. Even though NaCl and LiCl hyper tonic solutions cause nociceptive signals, only LiCl acts on the area postrema of the hindbrain, a structure associated with distress in the upper gastrointestinal tract (Adachi et al. 1991; Tsukamoto and Adachi 1994) producing a learned taste aversion in one trial (Garcia and Koelling 1967; Rabin and Hunt 1983). The behavioral response to lithium administration has been characterized as a general suppression of activity as well as a specific response described as “lying-on-belly,” diarrhea and urination, which are not present when a NaCl solution is administered (Garcia and Koelling 1967). In the case of dopamine, it has been reported that stressful stimuli, such as handling, induce an increase of dopamine release in the amygdala (Inglis and Moghaddam 1999). However, the lack of dopaminergic response during the i.p. administration may be due to the previous habituation of the animals to be handled; in fact, they did not show any signs of stress or discomfort at this time point. In summary, our results show that both norepinephrine and glutamate, but not dopamine, signal the gastric toxicity of the LiCl, whereas the norepinephrine release may be related to the stress response induced by drinking the novel taste.

After being engaged in the acquisition of CTA, we found that amygdala reactivation involves concomitant noradrenergic and glutamatergic signals ~45 min post-acquisition, but dopamine levels did not change during this period. Interestingly, only the association between novel taste and LiCl produced both glutamate and norepinephrine reactivation release at 45 min. Whereas the rest of the combinations—saccharin–NaCl, H2O–LiCl, or the backward presentation of stimuli—did not produce significant release of norepinephrine or glutamate at the 45-min post-acquisition period. It is noteworthy that even though the norepinephrine signals are very similar in the saccharin–LiCl and saccharin–NaCl groups, only the saccharin–LiCl had the key aversive signal of glutamate and showed the post-acquisition norepinephrine and glutamate increments. In other words, it seems that the combination of saccharin and LiCl in the forward fashion will enable the amygdala reactivation. Similar results have been found in the IC for dopamine instead of norepinephrine and glutamate—that is, only when saccharin–LiCl pairing was presented to the animals did we observe a concomitant release of both glutamate and dopamine in the IC, around 40 min after the association of the stimuli (Guzmán-Ramos et al. 2010).

To demonstrate the functionality of these post-acquisition signals on memory consolidation, we administered NMDAR or β-adrenergic receptors antagonists at 30-min post-acquisition, i.e., before the putative neurotransmitters binding in their respective receptors at 45 min when the observed release of both norepinephrine and glutamate occurred. The results indicated that post-acquisition blockade of the NMDAR and β-adrenergic receptors at 45-min impair CTA memory consolidation. Some reports support the notion that amygdalar NMDAR and β-adrenergic receptors play an essential role in CTA consolidation (Tucci et al. 1998; Yasoshima et al. 2000; Miranda et al. 2003): however, such blockades were made between the CS and the US presentation, affecting US processing and impairing CTA acquisition. Here we present evidence that suggest these receptors have to be reactivated after the learning period in order to consolidate CTA memory. Since the pharmacological treatments that we administered were performed after the exposure to the CS and US, and these treatments had effect only in the LTM, it indicates a specific role of the post-learning release of these neurotransmitters in taste memory consolidation. It has been reported that the modulation of the amygdala over the IC is important for CTA consolidation. For instance, long-term potentiation induced in the BLA–IC pathway produces significant CTA memory enhancement (Escolar and Bermudez-Rattoni 2000) which depends on NMDAR activation within the IC (Escolar et al. 2002). It has also been shown that the glutamatergic activity in the amygdala during acquisition modulate the NMDAR activity in the IC at least for an hour after the pairing of the stimuli (Ferreira et al. 2005). Part of the results in the present study could explain why this could be happening: the amygdala becomes active even after the learning period through β-adrenergic and NMDAR receptors to modulate the IC post-acquisition activity. In fact, the modulation exerted by the amygdala norepinephrine in post-acquisition stages has been well documented in other aversive tasks, such as inhibitory avoidance and contextual fear conditioning, where post-training intraamygdalar infusions of norepinephrine or clenbuterol (β-agonist) enhance memory consolidation (Introini-Collison et al. 1991, 1996; Izquierdo et al. 1992; Ferry and McGaugh 1999; Lalumiere et al. 2003; Lalumiere and McGaugh 2005). Herein, we provide evidence that in the CTA paradigm a post-acquisition activity takes place and is involved specifically in consolidation.

The actions of norepinephrine in memory consolidation may involve the activation of β-adrenergic receptors and stimulation of the cyclic AMP-dependent protein kinase (PKA)/cAMP response element binding (CREB) pathway (Liang et al. 1990; Ferry et al. 1999). PKA has been involved in regulating memory consolidation by its effects on ionic channels and ionotropic receptor conductance, particularly the NMDAR. Thus, the NMDAR are phosphorylated on the NR1 subunit, increasing the channel conductance that promotes a rise on postsynaptic Ca2+ (Tingley et al. 1997; Skerbids et al. 2006), which in turn activates kinases and phosphatases that promote persistent changes in synaptic strength. All of this information suggests that during the post-acquisition period, NMDAR and β-adrenergic receptors act in parallel to support the consolidation process. Moreover, it has also been reported that β-adrenergic receptors facilitate excitatory synaptic transmission in amygdalar pyramidal cells, so it is possible that a synergic activity could be taking place as a result of the concomitant release of norepinephrine and glutamate. The administration of a β-adrenergic agonist, such as isoproterenol, enhanced the evoked excitatory postsynaptic currents mediated by NMDAR, and this enhancement was blocked by application of Rp-adenosine 3′,5′-cyclic monophosphorothioate trethylammonium salt (Rp-cAMPS), a potent PKA inhibitor (Gean et al. 1992; Huang et al. 1993, 1998; Huang and Gean 1995). Therefore, the concomitant post-acquisition increase of norepinephrine and glutamate may activate both β-adrenergic and NMDAR receptors, enhancing the amygdala activity and promoting the flow of biochemical events involved in the consolidation process.

Although further studies are needed to prove the previous hypothesis, the data herein exposed indicate the relevance of the post-acquisition glutamatergic and noradrenergic activity in the amygdala. Thus, it could be possible that the amygdala needs to be reactivated after the acquisition of the task to outline the memory trace, which is consistent with the evidence that post-training brain activity is related to previous learning experience.
(Peigneux et al. 2006; Eschenko and Sara 2008; Lansink et al. 2008; Marrone et al. 2008). Particularly in the amygdala, single-unit recordings of spontaneous activity revealed that the firing rate of BLA neurons increased gradually after inhibitory avoidance training (tone paired with footshock), peaking at 30–50 min post-shock (Pelletier et al. 2005), a time frame that goes in accordance with the neurochemical reactivation herein described. Taken together, these results support the idea that keeping emotional experience in the long term requires post-acquisition amygdala activity. In summary, our results indicate that CTA memory consolidation involves amygdala post-acquisition glutamatergic and noradrenergic activity. The concomitant release of these neurotransmitters may imply a synergic action of the β-adrenergic and the NMDA receptors, enhancing neuronal activity and promoting plastic changes that are thought to underlie memory consolidation.

Materials and Methods

Subjects

Adult male Wistar rats (bred in the Veterinarian facilities of The Institute of Cellular Physiology, Mexico), weighing 260–280 g at the time of surgery, were used and handled according to The Institute of Cellular Physiology Animal Welfare Assurance, approved by NIH in accordance with the Norma Oficial Mexicana (NOM-062-ZOO-1999) and the European Communities Council Directive of November 24th 1986 (86/609/ECC). Rats were housed individually and maintained on a 12-h light/12-h dark cycle with water and food ad libitum, except when noted on the behavioral procedures. Training was conducted during the light portion of the cycle.

Guide cannulae implantation

Animals were anesthetized with a mixture of ketamine/xylazine (50 mg/kg + 5 mg/kg) and placed in a stereotaxic apparatus (Stoelting). A small incision in the scalp was made and the head position was adjusted to the horizontal plane. Unilateral guide cannula (CMA Microdialysis) aimed at the amygdala was implanted using coordinates from bregma according to Paxinos and Watson (1998) (AP –2.8 mm; L –4.8 mm; DV –7.5 mm). The cannula was held in place with two small screws and dental cement. For the drug administration experiments, bilateral 23-gauge stainless-steel cannulae (12-mm-long, small parts) were implanted aiming to the amygdala (AP –2.8 mm; L ± 4.8 mm; DV –6.5 mm). Dummy cannulae (33-gauge, 12 mm) were inserted into the guide cannulae to prevent clogging. Rats were allowed to recover from the anesthesia before being returned to their home cages.

Microdialysis procedure for CS and US associative presentation

Five days after surgery, animals were deprived of water for 24 h and were placed in the microdialysis chamber for 5 d during 3 h to habituate them to the environment and manipulation. All animals were allowed to drink 10 mL of tap water from a graded bottle during 15 min and another 20 mL in their home cages in the afternoon. During microdialysis, a dialysis probe with a 1-mm membrane (CMA 12 MD Probe, CMA Microdialysis) was connected to the micro infusion pump system (CMA Microdialysis), which infused artificial cerebrospinal fluid (ACSF; NaCl 125 mM, KCl 5 mM, NaH2PO4H2O 1.25 mM, MgSO47H2O 1.5 mM, NaHCO3 26 mM, CaCl2 2.5 mM, glucose 10 M) at a rate of 1 μL/min. The length of the outflow connection tubing was measured to calculate the “dead volume” (inner volume of the tubing × length of the tubing × infusion rate), which causes a delay between the beginning of the behavioral response and the correspondent microdialysis fraction. After insertion of the probe, 1 h of fluid stabilization was allowed; samples were collected at a rate of 0.8 μL/min every 5 min in vials containing 0.5 μL of antioxidant mixture (0.25 mM ascorbic acid, Na2EDTA 0.27 mM, 0.1 M acetic acid), and immediately frozen at ~80°C. Three fractions were collected as baseline samples before the stimulus exposure began (see below for behavioral conditions).

Analysis of glutamate and norepinephrine in microdialysate samples

The neurotransmitter concentration were determined by capillary electrophoresis as described previously (Guzmán-Ramos et al. 2010). Briefly, the procedure for microdialysis samples analysis was derivatization with 6 μL of 16.67 M 3-(2-furyl)quinoine-2-carboxaldehyde (FQ, Molecular Probes, Invitrogen) in the presence of 2 μL of KCN 23 mM in 10 mM borate buffer (pH 9.2) and 1 μL of internal standard (0.075 mM O-methyl-L-threo- nine, Sigma-Aldrich). The mixture was allowed to react in the dark at 65°C for 15 min. Separation and analysis were conducted in a Capillary Electrophoresis system (Beckman-Coulter PACE/MDO, Glycoprotein System) with laser-induced fluorescence detection, light at 488 nm from an argon ion laser was used to excite the FQ-labeled analytes. The separation was based on a micellar electrokinetic chromatography buffer system that included 35 mM borates, sodium dodecylsulphate 25 mM, 13% (vol/vol) methanol HPLC grade (final pH 9.6). The samples were injected hydrodynamically at 0.5 psi for 5 sec in a 75-μm i.d. capillary (Beckman Coulter), then the separation was performed at 25 kV. After each sample run, the capillary was flushed with 0.1 M NaOH, water and running buffer. The glutamate and norepinephrine peaks were identified by matching the migration pattern with those in a spiked sample and corrected by relating the area under the curve (AUC) of the unknown sample with the AUC of the internal standard. Data were analyzed using Karat System Gold (Beckman Coulter), generating a calibration curve for six points. All results were converted into percentage of baseline release (% BL = analyte concentration × 100/mean of the three first samples).

Behavioral procedure for drugs administration experiments

Five days after cannulae implantation, animals were deprived of water for 24 h and in the subsequent 5 d they were allowed to drink 70 mL of tap water for 15 min in the morning and 4.5 h later for another 15 min in order to perform a short-term memory (STM) and LTM test when required. This protocol for testing STM and LTM in the same animals has demonstrated that the saccharin presentation during STM has no interference with the LTM aversion response (Ferreira et al. 2005; Guzmán-Ramos et al. 2010). The volume of water ingested was measured, and the mean of this intake was considered as baseline. For CTA acquisition, rats were exposed to 0.1% (wt/vol) saccharin solution for 15 min, and 15 min later they received a 0.4 M LiCl i.p. injection (7.5 mL/kg). The STM was evaluated 4.5 h after the acquisition by re-exposing the rats to the same saccharin solution and registering the consumption; then LTM was evaluated 72 h after acquisition. Data are presented as percentage of consumption of saccharin during the test related to the saccharin consumption during acquisition (% Consumption during acquisition = saccharin solution intake during test × 100/saccharin solution intake during CTA acquisition).

Drug administration

All drugs were dissolved in saline solution (0.9% wt/vol). Drug administration was made through 30-gauge dental needles that protruded 1.5 mm from the tip of the guide cannulae. Injection needles were connected via polyethylene tubing to two 10-μL Hamilton syringes, driven by an automated micro infusion pump (Carnegie Medicine). A total volume of 1 μL per hemisphere was delivered at a rate of 0.5 μL/min. After micro infusions were completed, the injection needles were left in the guide
cannulae for an additional minute to allow diffusion of the solutions into the tissue. Animals were divided into three groups, counterbalanced according to their baseline mean consumption: The control group (n = 16) received saline solution microinjections; the APV group (n = 15) received DL-2-Amino-5-phosphonovaleric acid (APV, 10 μg/μL; Tocris Bioscience), and the PROP group (n = 21) received Propranolol infusions (5 μg/μL Sigma-Aldrich). All groups received the intra-amygdalar microinjections 30 min after the CTA training and were evaluated for STM and LTM.

Histology

After the experiments were completed, rats were sacrificed with a pentobarbital overdose, and perfused intracardially with saline 0.9%. Brains were removed and stored in paraformaldehyde 4% at 4°C. They were cut in 40-μm coronal slices with a freezing cryostat. Mounted slices were stained with cresyl violet, and the placement of the cannulae was verified by analyzing the slices under the light microscope. All animals with an erroneous cannulae placement were discarded from statistical analysis and final n-values were as described in each methodological section.

Statistical analysis

Statistical analysis of extracellular changes in the dialysates was carried out using two-way ANOVA with Fisher post-hoc test. ANOVA with post hoc Fisher test was used to analyze saccharin consumption data, a value of P < 0.05 was considered statistically significant. Statview 4 software (Abacus Concepts) was used to perform analysis and plots.

Acknowledgments

We thank Oreste Carbajal for the technical support. This work was supported by CONACyT 060748 and DGAPA UNAM IN216709-3.

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Received October 31, 2011; accepted in revised form March 20, 2012.
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Learn. Mem. 2012, 19:
Access the most recent version at doi:10.1101/lm.024703.111

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