Research

Memory trace reactivation and behavioral response during retrieval are differentially modulated by amygdalar glutamate receptors activity: interaction between amygdala and insular cortex

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The insular cortex (IC) is required for conditioned taste aversion (CTA) retrieval. However, it remains unknown which cortical neurotransmitters levels are modified upon CTA retrieval. Using in vivo microdialysis, we observed that there were clear elevations in extracellular glutamate, norepinephrine, and dopamine in and around the center of the gustatory zone of the IC during CTA retrieval. Additionally, it has been reported that the amygdala–IC interaction is highly involved in CTA memory establishment. Therefore, we evaluated the effects of infusions of an AMPA receptor antagonist (CNQX) and a NMDA receptor antagonist (APV) into the amygdala on CTA retrieval and IC neurotransmitter levels. Infusion of APV into the amygdala impaired glutamate augmentation within the IC, whereas dopamine and norepinephrine levels augmentation persisted and a reliable CTA expression was observed. Conversely, CNQX infusion into the amygdala impaired the aversion response, as well as norepinephrine and dopamine augmentations in the IC. Interestingly, CNQX infusion did not affect glutamate elevation in the IC. To evaluate the functional meaning of neurotransmitters elevations within the IC on CTA response, we infused specific antagonists for the AMPA, NMDA, DI, and β-adrenergic receptor before retrieval. Results showed that activation of AMPA, DI, and β-adrenergic receptors is necessary for CTA expression, whereas NMDA receptors are not involved in the aversion response.

Memory retrieval is the process in which the previously stored information is selected, accessed, reactivated, and reconstructed (Dudai 2002). It has been suggested that memory retrieval is a dynamic process whereby memory stored information could be modified and updated through post-retrieval processes similar to consolidation (Rodríguez-Ortíz et al. 2008; Flavell and Lee 2013; Garcia-Delatorre et al. 2014). Conditioned taste aversion (CTA) is an associative learning paradigm suitable for the study of neurobiological processes that underlie the mechanisms of acquisition, consolidation, and retrieval of memory. In CTA, animals associate a novel taste with gastric malaise; this association produces a decrease in the consumption of the gustatory stimulus in further presentations (García et al. 1955; Bermúdez-Rattoni 2004).

In rodents, the insular cortex (IC) is part of the cerebral cortex and is located at the confluence of the rhinal sulcus and the medial cerebral artery. The IC has been associated with the viscerosomatic information processing and has also been involved in several cognitive functions like memory formation of aversively and nonaversively motivated tasks (see Bermúdez-Rattoni 2004). In CTA paradigm, the IC has a key role in the brain circuitry that participates in the acquisition, consolidation, and retrieval of taste aversion memories (Bermúdez-Rattoni and McGaugh 1991; Guzmán-Ramos et al. 2010; Desgranges et al. 2009). During CTA acquisition, the exposure to a novel gustatory stimulus induces an elevation in acetylcholine (Miranda et al. 2000) and dopamine extracellular levels within the IC (Guzmán-Ramos et al. 2010). The induction of gastric malaise, by an intraperitoneal injection of LiCl, causes an augmentation in glutamate and norepinephrine concentrations within the IC (Guzmán-Ramos et al. 2010). Interestingly, there is a concomitant increase in dopamine and glutamate after CTA acquisition within the IC, these increments of glutamate and dopamine have a key role in the consolidation of taste aversion memory (Guzmán-Ramos et al. 2010).

It has been suggested that the functional integrity of the IC is required for CTA retrieval. For instance, expression of CTA during retrieval can be attenuated by the infusion of tetrodotoxin into the IC (Gallo et al. 1992) or by electrolytic (Cubero et al. 1999) and excitotoxic lesions of the IC (Schier et al. 2016). Additionally, it has been shown that in a multiple choice test, the activation of AMPA receptors within the IC is necessary to elicit CTA expression (Berman et al. 2000). Moreover, it has been demonstrated that the glutamatergic receptors activity has a fundamental participation in CTA retrieval and memory updating (Rodríguez-Ortíz et al. 2012; Garcia-Delatorre et al. 2014; Osorio-Gómez et al. 2016). Altogether, these results clearly suggest that glutamate in the IC is involved in CTA retrieval. However, there is scarce information about the other neurotransmitters involvement during CTA retrieval.

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The IC and the amygdala are highly interconnected and this interaction participates in CTA memory formation. In this regard, the tetanic stimulation of the basolateral amygdala induces long-term potentiation in the IC through NMDA receptors activity (Escobar et al. 1998). This long-term potentiation induction enhances the retention of taste aversion memory (Escobar and Bermúdez-Rattoni 2000). During the CTA acquisition, glutamate in the amygdala is modulating the strength of CTA memory through the activation of NMDA receptors within the IC (Ferreira et al. 2005). Also, it has been demonstrated that during CTA post-acquisition phase, the infusion of tetrodotoxin into the amygdala causes an impairment of CTA consolidation and reduces the concomitant augmentation of dopamine and glutamate levels within the IC (Guzmán-Ramos et al. 2010). Moreover, it has been shown that after CTA learning, there is an increase in the functional connectivity between the amygdala and the IC (Grossman et al. 2008). Given the importance of amygdala–IC connectivity in the formation of taste aversive memory, it is relevant to determine the participation of the amygdala communication with the IC during memory retrieval.

In the first part of this study, we measured the extracellular levels of glutamate, norepinephrine, and dopamine in and around the center of the gustatory zone of the IC during CTA retrieval. In addition, to test the hypothesis that neurotransmission in the IC could be modulated by the activity of glutamatergic receptors in the amygdala; we infused an AMPA receptor antagonist (CNQX) and a NMDA receptor antagonist (APV) into the amygdala on CTA retrieval while microdialysis in the IC was carried out. Finally, we evaluated the functional role of glutamate, norepinephrine, and dopamine in the IC on CTA retrieval by the infusion of specific antagonists for the AMPA (CNQX), NMDA (APV), D1 (SCH-23390), and β-adrenergic (Propranolol) receptors before retrieval.

Results

To evaluate changes in the extracellular levels of glutamate, norepinephrine, and dopamine in the IC during CTA retrieval, we used in vivo microdialysis during and after the exposure to the conditioned stimulus. The animals were divided into two groups, the aversive group (Aversive, $n = 8$), which was previously exposed to 30 mL of a 0.1% (wt/vol) sodium saccharin solution, followed by an i.p. LiCl injection (0.2 M, 7.5 mL/kg) and the nonaversive group (Nonaversive, $n = 7$), which was previously exposed to the same saccharin solution, but later it received an i.p. NaCl injection (0.2 M, 7.5 mL/kg), that does not cause taste aversion. Microdialysis was carried out in both groups throughout CTA retrieval where animals were exposed to 0.1% sodium saccharin solution.

Extracellular levels of glutamate within IC during CTA retrieval

Statistical analysis indicated significant differences in the extracellular levels of glutamate between groups ($F_{(1,120)} = 11.238$ $P < 0.01$), among fractions ($F_{(5,120)} = 2.676$ $P < 0.05$) and a group–fractions interaction effect was observed ($F_{(5,120)} = 3.300$ $P < 0.01$). The Aversive group showed differences in glutamate levels compared with the basal concentration at the sixth fraction ($P < 0.01$), where the aversive stimulus was present, whereas the Nonaversive group revealed no significant differences in glutamate levels regarding the presentation of saccharin. Figure 1B shows changes on the extracellular levels of glutamate in the IC during CTA retrieval.

Extracellular levels of norepinephrine within IC during CTA retrieval

In relation to norepinephrine, the statistical analysis revealed significant differences in the extracellular levels of norepinephrine within the IC between groups ($F_{(1,115)} = 4.343$, $P < 0.05$) and among fractions ($F_{(5,115)} = 5.253$, $P < 0.01$), whereas no interaction group–fraction effect was observed ($F_{(5,115)} = 1.559$, $P =$ NS) during CTA retrieval. In the Aversive group, there was an augmentation of the extracellular levels of norepinephrine at the sixth fraction when the rats were exposed to the aversive stimulus ($P < 0.01$); while no significant changes between fractions were observed in the Nonaversive group. Figure 1C displays changes of the extracellular levels of norepinephrine within the IC during CTA retrieval. Our results showed that in aversive conditioned animals, there was a clear augmentation in norepinephrine concentration in the IC when the aversive conditioned stimulus was present; whereas the animals that were exposed to a nonaversive saccharin did not show an increase in noradrenergic concentration in the IC.

Extracellular levels of dopamine within IC during CTA retrieval

Regarding dopamine in the IC, there were significant differences between groups ($F_{(1,124)} = 8.625$ $P < 0.01$); among fractions ($F_{(5,124)} = 4.684$ $P < 0.01$); and no interaction group–fraction effect was observed ($F_{(5,124)} = 1.890$ $P =$ NS). The Aversive group showed an increase in dopamine levels compared with the basal concentration at the sixth fraction when saccharin was presented ($P < 0.01$), while in the Nonaversive group, the exposure to saccharin did not alter the levels of dopamine in the IC. Figure 1D shows changes of the extracellular levels of dopamine during retrieval.

CTA behavioral response results

Conditioned taste aversion response was estimated by measuring the consumption of saccharin during microdialysis. Nonpaired Student's *t*-test showed that the Aversive group displayed a significant and clear taste aversion, whereas the Nonaversive group failed to elicit reliable CTA ($t = 7.135$, $P < 0.01$) (Fig. 1E).

Correlation between aversion response and neurotransmitter elevation

A Spearman correlation coefficient was calculated to assess the relationship between the saccharin consumption and glutamate elevation at the sixth fraction. There was a negative correlation between the two variables ($r = -0.648$, $P < 0.05$). Regarding norepinephrine, we observed a negative correlation between the saccharin consumption and norepinephrine elevation at the sixth fraction ($r = -0.672$, $P < 0.05$). However, we did not observe a correlation between aversion response and dopamine extracellular levels within the IC ($r = -0.291$, $P =$ NS).

According to these results, the presentation of saccharin associated with gastric malaise induced a clear taste aversion response and an augmentation in the extracellular glutamate, norepinephrine, and dopamine in the IC during CTA retrieval. The increased glutamate and norepinephrine in the IC is related to the diminished consumption of the aversive taste stimulus during retrieval.

Given the previous results, we evaluated the hypothesis that neurochemical changes in the IC could be modulated by the activity of glutamatergic receptors in the amygdala, hence, we infused an AMPA receptor antagonist (CNQX 1 mg/mL, $n = 9$), a

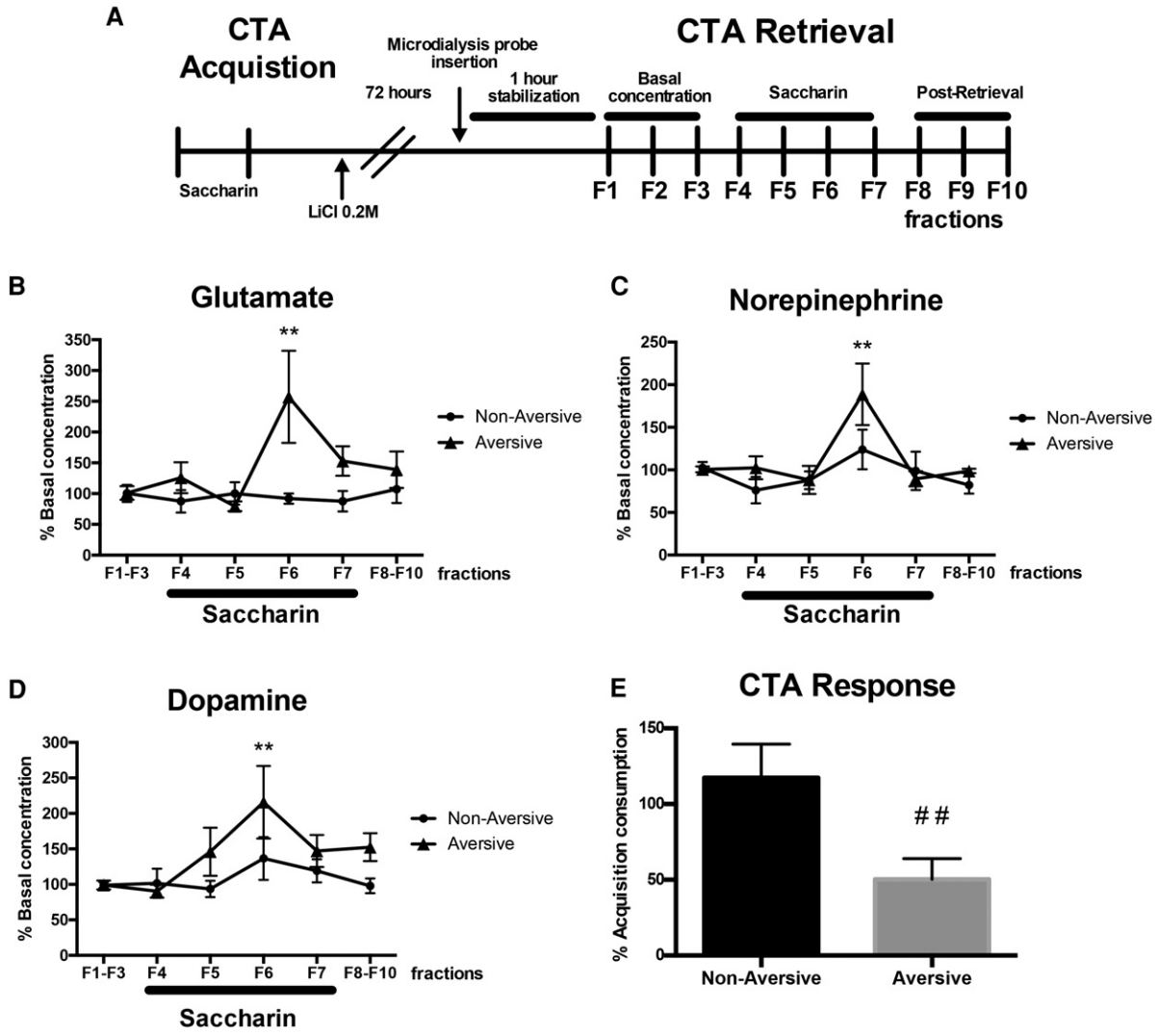

Figure 1. Behavioral CTA responses and extracellular levels of glutamate, norepinephrine, and dopamine in the insular cortex during CTA retrieval. (*A*) Schematic representation of the microdialysis protocol used. Changes in (*B*) glutamate, (*C*) norepinephrine, and (*D*) dopamine levels in conditioned animals (Aversive, $n = 8$) and nonconditioned animals (Nonaversive, $n = 7$) during CTA retrieval. The aversive conditioned stimulus elicits an augmentation in glutamate, norepinephrine, and dopamine in the Aversive group upon CTA retrieval. (*E*) The Aversive group displays a reliable taste aversion during memory retrieval. Graphics are expressed as means of percentage of basal concentration ± SEM. (**) $P < 0.01$ versus basal concentration. Behavioral response graph is expressed as the mean of percentage of saccharin consumption intake during CTA acquisition ± SEM. (##) $P < 0.01$ versus Nonaversive group.

NMDA receptor antagonist (APV 10 mg/mL, $n = 8$), or saline solution (SS, $n = 9$) into the amygdala before exposure to the aversive conditioned saccharin while performing microdialysis in the IC during CTA retrieval.

Differential effects of AMPA and NMDA activity in the amygdala over neurotransmitter levels within the IC during retrieval

Statistical analysis indicated that extracellular levels of glutamate in the IC changed among fractions ($F_{(5,217)} = 5.175$, $P < 0.01$) and a group–fraction interaction effect was observed ($F_{(10,217)} = 1.928$, $P < 0.05$). No differences were observed among groups (SS, CNQX, APV) ($F_{(2,217)} = 0.774$, $P =$ NS). Post hoc analysis revealed that glutamate concentration in the IC changed among fractions in animals infused with SS ($F_{(5,81)} = 4.819$, $P < 0.01$) or CNQX ($F_{(5,68)} = 2.991$, $P < 0.05$) into the amygdala during CTA retrieval. Specifically, there was an augmentation at the sixth fraction ($P < 0.01$) when the aversive conditioned stimulus was presented. Conversely, the extracellular levels of glutamate in the IC were similar among fractions in the APV group upon CTA retrieval ($F_{(5,68)} = 1.542$, $P =$ NS) (Fig. 2B,C).

Regarding norepinephrine, the extracellular levels in the IC varied among fractions ($F_{(5,222)} = 5.215$, $P < 0.01$) and a group–interaction effect was observed ($F_{(10,222)} = 2.274$, $P < 0.05$). No differences were observed among groups ($F_{(2,222)} = 1.353$, $P =$ NS). Post hoc analysis showed that extracellular levels of norepinephrine within the IC varied among fractions in the groups infused with SS ($F_{(5,80)} = 2.274$, $P < 0.01$) or APV ($F_{(5,68)} = 2.899$, $P < 0.05$) into the amygdala during CTA retrieval. Specifically, there was an increase at the sixth fraction in the SS group ($P < 0.01$) and at the fifth fraction in the APV group ($P < 0.05$). On the other hand, post hoc analysis showed that extracellular levels of norepinephrine within the IC were similar among fractions in

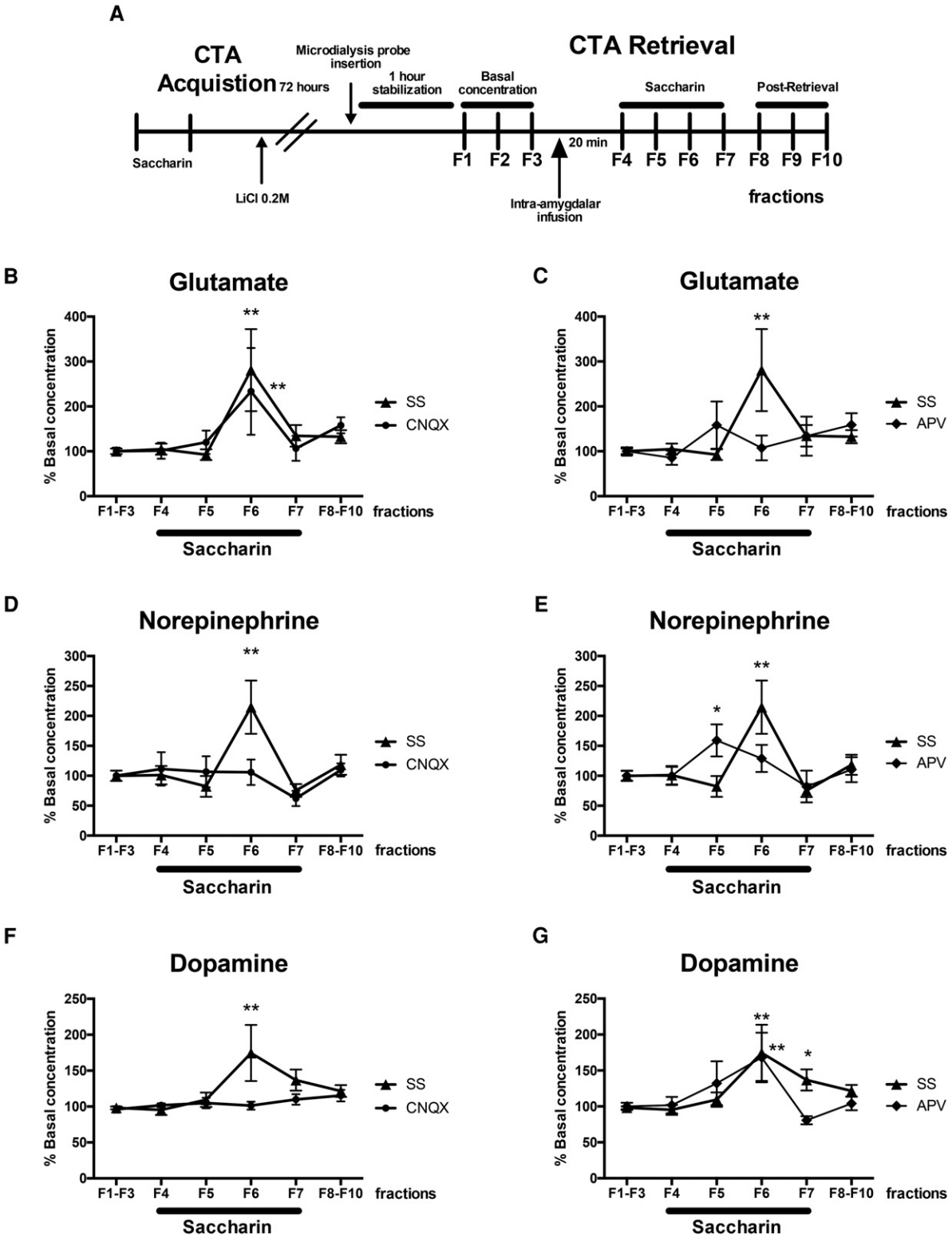

Figure 2. Glutamate receptors in the amygdala differentially affect neurotransmitter levels increase in insular cortex. (*A*) Schematic representation of the microdialysis and intra-amygdalar infusion protocol used. (*B*) The infusion of CNQX into the amygdala does not affect glutamate increase in the IC during CTA retrieval (CNQX, $n = 9$). (*C*) The infusion of APV into the amygdala impaired glutamate augmenation in the IC during CTA retrieval (APV, $n = 8$). (*D*) Norepinephrine extracellular levels in the IC during CTA retrieval are hindered by CNQX in the amygdala. (*E*) APV infusion into the amygdala does not affect norepinephrine increase within IC when the aversive stimulus is present. (*F*) Extracellular levels of dopamine in the IC during CTA retrieval are diminished due to CNQX infusion into the amygdala. (*G*) Infusion of APV into the amygdala does not have an effect on dopamine increase within IC when the aversive stimulus is present. Graphics are expressed as means of percentage of basal concentration ± SEM. (*) $P < 0.05$ and (**) $P < 0.01$ versus basal concentration.

the group injected with CNQX in the amygdala ($F_{(5,74)} = 1.140$, $P =$ NS) (Fig. 2D,E).

The statistical analysis indicated that extracellular levels of dopamine in the IC were different among fractions ($F_{(5,220)} = 5.831$, $P < 0.01$), among groups ($F_{(2,220)} = 3.468$, $P < 0.05$) and a group–fraction interaction effect was observed ($F_{(10,220)} = 2.380$, $P < 0.05$). Post hoc analysis revealed that extracellular levels of dopamine in the IC changed among fractions in the groups infused with SS ($F_{(5,77)} = 3.930$, $P < 0.01$) and APV ($F_{(5,70)} = 3.594$, $P < 0.01$) into the amygdala during CTA retrieval. This augmentation was observed at the sixth fraction ($P < 0.01$) in the presence of the aversive conditioned stimulus. Conversely, dopamine concentrations within the IC were similar among fractions in the animals infused with CNQX into the amygdala during CTA retrieval ($F_{(5,73)} = 3.594$, $P =$ NS) (Fig. 2F,G).

Differential effects of AMPA and NMDA activity in the amygdala on CTA behavioral response

Statistical analysis revealed that there were significant differences in saccharin consumption during CTA retrieval among groups ($F_{(2,22)} = 25.755$, $P < 0.01$). Post hoc analysis showed that the animals injected with CNQX into the amygdala displayed a reduced aversion response compared with SS or APV groups ($P < 0.01$), meanwhile no differences were observed between the SS and APV groups ($P =$ NS) indicating that both groups elicited a reliable CTA expression (Fig. 3).

Our results indicate that blockade of AMPA receptors in the amygdala hindered CTA expression, as well as hampered the augmentation of norepinephrine and dopamine extracellular levels in the IC during CTA retrieval; however, the increase in glutamate levels persisted. Conversely, the blockade of NMDA receptors in the amygdala disrupted the augmentation of glutamate within the IC while the increased catecholaminergic levels and CTA expression remained intact.

To evaluate the functional role of catecholamines and glutamate in the IC on CTA response, we infused selective antagonists of NMDA, AMPA, dopamine D1, and β-adrenergic receptors (with APV, CNQX, SCH-23390 and propranolol, respectively) into the IC 20 min before CTA retrieval and we measured the aversion displayed by the animals.

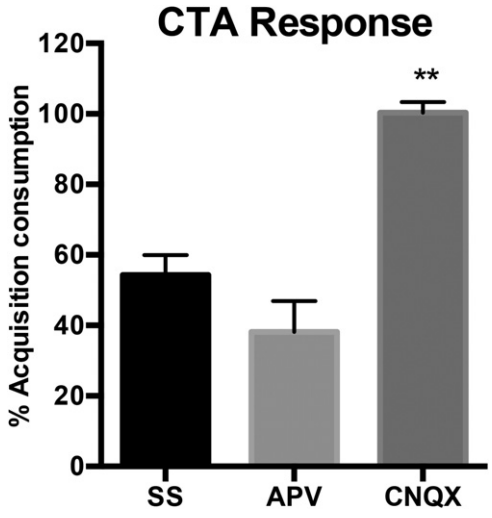

Figure 3. Behavioral CTA responses during retrieval. The infusions of saline solution and APV into the amygdala do not hinder a reliable aversion during CTA memory retrieval, whereas injection of CNQX into the amygdala impairs CTA response. Graphics are expressed as percentage of of acquisition consumption ± SEM. (**) $P < 0.01$ versus saline solution group.

Pharmacological disruption of CTA behavioral response in the IC

The one-way ANOVA analysis showed differences in the consumption of saccharin during CTA retrieval among groups ($F_{(4,27)} = 0.4519$, $P < 0.001$). Post hoc analysis showed that infusions of CNQX ($P < 0.01$, $n = 7$), Propranolol ($P < 0.01$, $n = 7$) or SCH ($P < 0.01$, $n = 6$) into the IC generate a reduced aversion response compared with SS ($n = 6$) or APV ($n = 6$) groups during retrieval, meanwhile no differences were observed between SS and APV groups ($P =$ NS) indicating that both groups elicit a reliable CTA expression (Fig. 4). Our results indicate that catecholaminergic activity in the IC, through dopamine D1 and β-adrenergic receptors activation, is necessary for CTA response. Regarding glutamate, the NMDA, and AMPA receptors have differential effects on retrieval, the AMPA receptors blockade in the IC impairs CTA expression whereas NMDA receptors blockade does not induce deficits in taste aversion response.

Verification of cannula placement

Coronal sections (40 μm) were cut and stained with cresyl violet. We observed the placement of the microdialysis probes for the Aversive group and the Nonaversive group in the IC region (Fig. 5A). In the amygdala–IC interaction experiment (Fig. 5B), the location sites for the injector tips were between the central and the basolateral amygdala, the infused volume of drugs (1 μL) is sufficient to reach both nuclei. In the pharmacological blockade experiments, the injector tips were observed in the IC area (Fig. 5C). Ten animals from the following groups: Aversive = 1, Nonaversive = 2, CNQX-Amy = 1, APV-Amy = 2, SS-Amy = 1, CNQX-IC = 1, APV-IC = 1, SS-IC = 1) with misplaced cannulas were discarded from the statistical analysis.

Discussion

Although memory retrieval is a very important process, there is scarce information about the physiological mechanisms that subserve the selection, reactivation, and reconstruction of stored internal representations (Dudai 2002). The microdialysis technique is a powerful tool to determine neurotransmitter levels in mnemonic processes. The insular cortex is not functionally homogeneous and is a long structure of which only a portion is involved with taste processing (Lin et al. 2015) and although we positioned the microdialysis probe in the center of the gustatory zone, we cannot dismiss the possibility that a portion of the collected fractions were derived from surrounding sites. Nevertheless, the effects we report here were taste-dependent exclusively.

During CTA acquisition, the presentation of a novel taste stimulus does not induce cortical glutamatergic changes within the IC (Miranda et al. 2002; Guzmán-Ramos et al. 2010). However, the exposure to a novel taste stimulus induces an increment in acetylcholine (Miranda et al. 2000) and dopamine (Guzman-Ramos et al. 2010) extracellular levels in the IC. Nevertheless, we observed that the presentation of LiCl, used as unconditioned stimulus, produces an augmentation in glutamate levels within IC (Guzmán-Ramos et al. 2010). Here, we report that the presentation of a conditioned aversive taste stimulus can elicit an increment in extracellular levels of glutamate in the IC, similar to the augmentation of glutamate induced by i.p. LiCl observed during CTA acquisition (Guzmán-Ramos et al. 2010). These results suggest that during CTA retrieval, the consumption of a

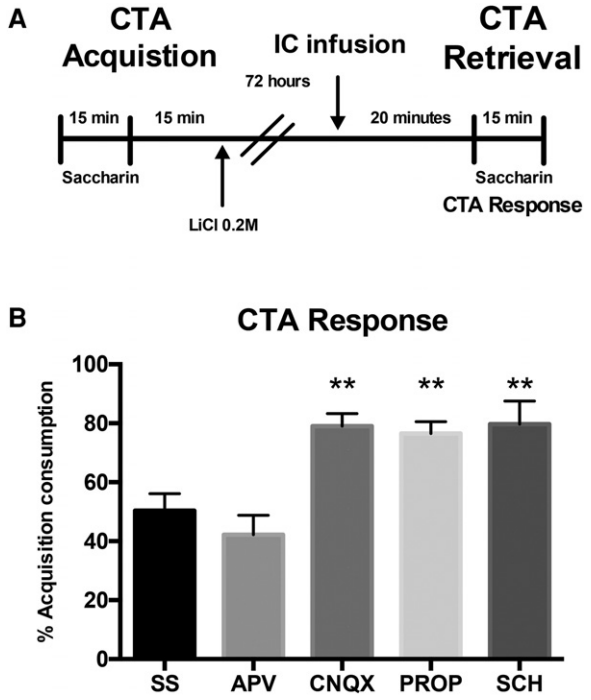

Figure 4. Pharmacological disruption of CTA response in the IC. (*A*) Schematic representation of the intra-cortical infusion protocol used during CTA retrieval. (*B*) The infusion of CNQX ($n = 7$), Propranolol (PROP, $n = 7$) or SCH ($n = 6$) into the IC before retrieval impairs CTA expression. The infusion of a NMDA antagonist into de IC (APV, $n = 6$) does not affect CTA response. Graphics are expressed as percentage of acquisition consumption ± SEM. (**) $P < 0.01$ versus saline solution group (SS, $n = 6$)

conditioned aversive taste stimulus induces a conditioned response measured as an augmentation of the extracellular levels of glutamate within the IC and a diminished intake of the stimulus.

In relation to catecholamines, it is widely known that norepinephrine is involved in the establishment of aversive memories (McGaugh et al. 2013). Presentation of stressful and relevant stimuli elicits norepinephrine release in the forebrain (Morilak et al. 2005), including the IC (Funk and Stewart 1996). In this regard, it has been reported that during CTA acquisition, the exposure to a novel gustatory stimulus and the i.p. injection of NaCl or LiCl induces an elevation of norepinephrine in the IC (Guzmán-Ramos et al. 2010). Therefore, novelty and noxious stimuli generate an elevation of norepinephrine in the IC, this augmentation is probably related to the relevance of the stimuli. During CTA retrieval, the exposure to the conditioned taste aversive stimulus is a relevant event because saccharin was previously associated with gastric malaise. Hence, the consumption of an aversive gustatory stimulus induces an augmentation in the extracellular levels of norepinephrine in the IC as a consequence of the relevance of the information retrieved.

It has been suggested that dopaminergic neurons respond to salient stimuli (Ljungberg et al. 1992; Ungless 2004). In this regard, the presentation of an aversive stimulus generates an augmentation of the dopaminergic neurons activity (Brischoux et al. 2009). Likewise, even the exposure to a novel stimulus induces an elevation of dopamine concentration within the IC (Guzmán-Ramos et al. 2010). Conjointly, when animals are exposed to a familiar taste, there is a reduction in the responsiveness of dopaminergic neurons in the nucleus accumbens as a result of habituation to the salient information (De Luca 2014). Therefore, an aversive conditioned stimulus induces an increase in dopamine levels within the IC, probably as a response to the salient stimulus, whereas the presentation of a nonaversive and familiar stimulus evokes a minor increase of dopamine due to a lack of salience.

Herein, the presentation of a conditioned taste aversive stimulus induces an elevation of glutamate, dopamine and norepinephrine concentrations within the IC. Although there is a clear diminished mean intake of the conditioned saccharin (around 6 mL), the augmentation of neurotransmitters is possibly related to the learned characteristics of the conditioned stimulus and not to the amount consumed; considering that even 5 mL of the taste stimulus is sufficient to induce an elevation of c-fos expression in the IC (Koh et al. 2003) and in the nucleus of the solitary tract, another important brain area involved in CTA (Swank et al. 1995). Furthermore, the expression of activity-regulated cytoskeleton-associated protein (Arc) is augmented in the IC due to the presentation of 5 mL of a familiar taste (Morin et al. 2011).

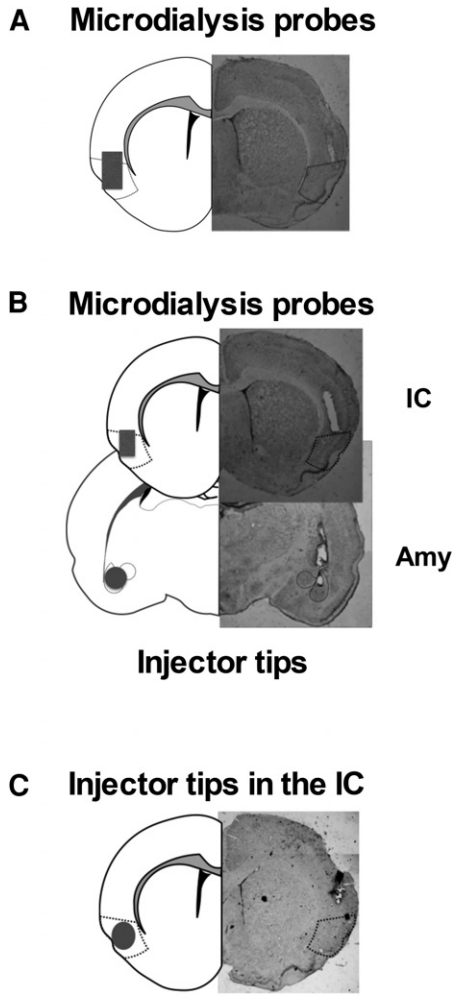

Figure 5. Schematic representation of the location of microdialysis cannula in the IC and the injector tips in the amygdala region. (*A*) Representative photomicrography of the IC with cannula trace and representation of probes location area. (*B*) Representative microphotographs of the amygdala with injector tip and microdialysis cannula trace location within the IC; and representation of probes and injector tips location areas. (*C*) Representative microphotographs of the IC with injector tip and representation of injector tips location areas.

It is important to stress that there are excitatory glutamatergic projections from the insula to the amygdala (Kobayashi 2011). However, our experiments were aimed to understand the modulation of the insular cortex by the activation of glutamate receptors in the amygdala. Nevertheless, we cannot entirely dismiss the possibility that the insular projections to the amygdala could have an important role during CTA retrieval. Additionally, it is important to mention that we cannot entirely discount the possibility that the infusions of the glutamate receptor antagonists targeting the amygdala could diffuse into adjacent structures. However, the effects reported here were taste dependent, orderly, robust, and consistent with what is known about the anatomical connectivity between the IC and the amygdala. In this study, the infusion of an AMPA antagonist in the amygdala reduces taste aversion response and hinders the augmentation of norepinephrine and dopamine extracellular within the IC. These results suggest that AMPA receptors in the amygdala are modulating CTA expression and the catecholaminergic extracellular levels within the IC. Interestingly, AMPA receptors blockade in the amygdala does not disrupt glutamatergic augmentation in the IC during CTA retrieval. Conversely, the infusion of a NMDA receptor antagonist into the amygdala does not disrupt CTA behavioral response, neither norepinephrine nor dopamine extracellular levels elevation within the IC are impaired. However, the blockade of NMDA receptors in the amygdala hinders the increase in glutamate concentration within the IC. Understanding the mechanisms that explain how the amygdala is modulating catecholaminergic changes within the IC is not an easy task. It is known that the amygdala has glutamatergic projections to the locus coeruleus (Luppi et al. 1995) and to the ventral tegmental area (Phillipson 1979; Geisler et al. 2007). Thereby, we suggest that the activation of AMPA receptors in the amygdala is modulating the CTA expression through glutamatergic efferent projections to the locus coeruleus and the ventral tegmental area, regulating catecholaminergic release in several brain structures including the IC.

Conjointly, the NMDA receptors in the amygdala are modulating the extracellular levels of glutamate in the IC. In this regard, we have previously observed that the temporal inactivation of the amygdala by tetrodotoxin infusions hindered the augmentation of glutamate within the IC (Guzmán-Ramos et al. 2010). Additionally, it has been reported that the amygdala–IC interaction is principally through glutamate and this interaction has a key role in the formation and maintenance of CTA memory. Tetanic stimulation of the amygdala induces long-term potentiation in the IC that depends on NMDA receptors activation (Escobar et al. 1998); this long-term potentiation enhances CTA memory retention (Escobar and Bermúdez-Rattoni 2000). Likewise, blockade of NMDA receptors in the IC impairs the enhancement of CTA obtained through glutamate injections in the amygdala (Ferreira et al. 2005). All these results suggest that the amygdala–IC interaction is mainly glutamatergic and has a key role in the formation and maintenance of CTA memory.

To evaluate the functional role of catecholamines and glutamate in the IC on CTA retrieval, we pharmacologically blocked the NMDA, AMPA, β-adrenergic and dopamine D1 receptors before CTA retrieval. The catecholaminergic activity, through D1 and β-adrenergic receptors activation, is involved in the expression of taste aversion. Moreover, the infusion of an AMPA receptor antagonist into the IC hindered taste aversion response, whereas the NMDA antagonist has no effect on CTA expression. In this regard, Berman et al. (2000; 2001) suggested that CTA expression during retrieval is independent of NMDA, dopaminergic D1, and β-adrenergic receptors activation; whereas AMPA receptors activation in the IC is necessary to elicit taste aversion in a multiple choice test. Although, our results are different, these discrepancies can be explained by the behavioral protocols used; during CTA retrieval we presented one bottle with saccharin while they performed a multiple choice test, presenting three bottles with saccharin and three bottles with water, promoting a strong aversion. Moreover, there is evidence that expression of aversive memories is impaired by the infusion of a β-adrenergic antagonist into the hippocampus (Barros et al. 2001; Murchison et al. 2004; Otis et al. 2014), the amygdala and the entorhinal, parietal, and anterior cingulate cortices (Barros et al. 2001). This impairment has also been induced by the infusion of a D1 dopaminergic antagonist (Barros et al. 2001). Therefore, a diminished catecholaminergic activity in the IC, through modulation of the amygdala or by pharmacological blockers, hinders CTA response during retrieval. These results clearly suggest that norepinephrine and dopamine augmentations in the IC are modulating the aversion response through dopamine D1 and β-adrenergic receptors activation during CTA retrieval.

In relation to glutamate, the functional role of AMPA receptors in the expression of memories has been widely described in the amygdala (Rodríguez-Ortíz et al. 2012; Garcia-Delatorre et al. 2014; Osorio-Gómez et al. 2016), perirhinal cortex (Santoyo-Zedillo et al. 2014), and the IC (Berman et al. 2000). Those reports and our results suggest that AMPA receptors activation is necessary to induce the expression of the learned response. Conversely, the behavioral expression of memories seems to be a process that does not require the NMDA receptors activation (Berman et al. 2000; Garcia-Delatorre et al. 2014; Santoyo-Zedillo et al. 2014; Osorio-Gómez et al. 2016). Accordingly, we report that the infusion of a NMDA receptor antagonist into the IC does not hamper the aversion response. Therefore, we suggest that NMDA receptors activity is not involved in the expression of taste aversion but instead trigger other memory events, like reconsolidation and information updating during CTA retrieval. In this regard, the activation of NMDA receptors is required for the establishment (Ferreira et al. 2005, Parkes et al. 2014), consolidation (Escobar and Bermúdez-Rattoni 2000; Guzmán-Ramos et al. 2010), and reconsolidation (Garcia-Delatorre et al. 2014) of CTA memory. Moreover, it has been observed that NMDA receptors activation in the IC is necessary to maintain the short taste memory trace for the ulterior association with gastric malaise (Adaikkan and Rosenblum 2015). Accordingly, we suggest that glutamate augmentation within the IC, regulated by the amygdala projections, has a key role in the taste memory trace reactivation to promote memory maintenance, updating and reconsolidation through NMDA receptors activity.

In summary, the activation of AMPA receptors in the amygdala is regulating the extracellular levels of norepinephrine and dopamine within the IC, these catecholamines are responsible for the expression of the learned response, whereas amygdalar interaction with the IC through glutamatergic communication could be involved in the taste memory trace reactivation facilitating memory maintenance and memory updating. Therefore, we show evidence that during retrieval, the behavioral expression is an independent process of memory trace reactivation (Ben Mamou et al. 2006; Flavell et al. 2011; Rodríguez-Ortíz et al. 2012; Coccoz et al. 2013; Balderas et al. 2013; Garcia-Delatorre et al. 2014; Santoyo-Zedillo et al. 2014; Delorenzi et al. 2014). Overall, our results suggest that memory retrieval is a process composed of independent signaling mechanisms that are necessary for behavioral expression and memory trace reactivation.

Materials and Methods

Animals

Eighty-three adult male Wistar rats weighing 260–280 g were used in this study. The animals were obtained from the Instituto de

Fisiología Celular and were housed individually at 22°C in a 12 h light/12 h dark cycle starting at 7 a.m., all procedures were performed during the light cycle and were approved by the animal care and ethics committee of the Instituto de Fisiología Celular (FBR 30-14). Water and food were ad libitum except during behavioral protocols.

Guide cannula implantation

Rats were implanted with a unilateral guide cannula (22 gauge, shaft length 14 mm. CMA Microdialysis) targeting the IC using standard stereotaxic procedures. Implantation of the cannula was according to the coordinates from Bregma (AP + 1.2 mm; L +5.5 mm; DV −4.5 mm) (Paxinos and Watson 1998). The cannula was fixed to the skull using two screws with dental acrylic cement. Right and left hemispheres were counterbalanced.

Microdialysis protocol

After 6 d of recovery, animals were water-deprived 24 h prior to behavioral procedures and then rats were habituated in a microdialysis chamber once a day for 1 h. Rats were allowed to drink 30 mL of tap water from a graded bottle during 15 min and baseline consumption was established over 6 d. On the seventh day, rats were separated into two groups, an aversion group (Aversive, $n = 8$), which was exposed to 30 mL of a 0.1% (wt/vol) sodium saccharin solution (SAC) (Sigma-Aldrich) during 15 min and followed by i.p. LiCl (Baker) injection (0.2 M, 7.5 mL/kg) 15 min after SAC consumption. In the control group (Nonaversive, $n = 7$), rats were exposed to SAC, but later they received an i.p. NaCl (Sigma-Aldrich) injection (0.2 M, 7.5 mL/kg) that did not cause gastric malaise and therefore no taste aversion would be developed. Three days after training, animals were tested and both groups underwent microdialysis. The recollection of dialysis fraction began with the insertion of a dialysis probe with a 3 mm length and 0.5 mm diameter membrane (CMA 12 MD Probe, CMA Microdialysis) connected to the micro-infusion pump system (CMA Microdialysis) which perfused the probe continuously at a rate of 1 μL/min with Ringer solution (NaCl 118 mM, KCl 4.7 mM, $NaH_2PO_4H_2O$ 1.2 mM, $MgSO_4H_2O$ 1.2 mM, $NaHCO_3$ 19 mM, $CaCl_2$ 2.5 mM, glucose 3.3 mM). After probe insertion, the first sampling hour was discarded due to fluid stabilization; samples were collected every 4 min (4 μL/sample) in vials containing 1 μL of an antioxidant mixture (0.25 mM ascorbic acid, Na_2EDTA 0.27 mM, 0.1 M acetic acid). Samples were immediately frozen at −80°C. The first three samples were used to calculate the extracellular basal concentration in the IC, afterward a graded bottle with SAC was placed in the microdialysis chamber for 15 min and consumption intake was measured, microdialysis sampling extended until 10 fractions were collected.

Analysis of microdialysate samples

Neurotransmitter concentrations were determined by capillary electrophoresis. Briefly, samples were derivatized with 6 μL of 16.67 mM 3-(2-furoyl)quinoline-2- carboxaldehyde (FQ, Molecular Probes, Invitrogen) in the presence of 2 mL of KCN 25 mM in10 mM borate buffer (pH9.2) and 1 μL of internal standard (0.075 mM *O*-methyl-L-threonine, Sigma-Aldrich). Derivatization reactions were carried out in the dark at 65°C for 15 min. Capillary electrophoresis-based separations with laser induced fluorescence detection were used for analysis (Beckman-Coulter PACE/ MDQ, Glycoprotein System). Mixture separation was based on micellar electrokinetic chromatography buffer system. Glutamate, norepinephrine, and dopamine peaks were identified by matching electropherograms with a spiked sample. Samples were corrected by relating the area under the curve of the unknown sample with the area under the curve of the internal standard. Analyses were performed with the Karat System Gold software (Beckman Coulter). Results are expressed as percentage of basal concentration (percentage of Basal concentration = (analyte concentration × 100/mean of the three first samples).

Pharmacological manipulation of the amygdala during CTA retrieval while monitoring neurotransmitter changes within the IC

Unilateral microdialysis cannula (22 gauge, shaft length 14 mm. CMA Microdialysis) aiming the IC (AP + 1.2 mm; L +5.5 mm; DV −4.5 mm) and bilateral steel cannulas (12 mm length and 23 gauge) aiming the amygdala (AP −2.8 mm, L ± 4.8 mm, DV −6.5; Guzmán-Ramos et al. 2010) were implanted using standard stereotaxic protocols. The behavioral scheme was performed as described above. During CTA acquisition all animals were exposed during 15 min to 30 mL of SAC in a graded bottle and 15 min afterwards they received an i.p. LiCl injection (0.2 M, 7.5 mL/kg). CTA retrieval was carried out 72 h after acquisition. Rats were divided into three groups counterbalanced according to their acquisition mean consumption. Microdialysis started by inserting a dialysis probe with a 3 mm length membrane in the IC, connected to the microinfusion pump system, which perfused ringer solution at a rate of 1 μL/min. The first sampling hour was discarded and samples were collected every 4 min (4 μL/sample) in vials containing 1 μL of an antioxidant mixture. After three samples were collected, an injector was inserted into each guide cannula aiming to the amygdala extending 1 mm below the tip, injection needles were connected via polyethylene tubing into 10 μL Hamilton syringes, driven by an automated micro-infusion pump (Carnegie Medicine). A volume of 1 μL (0.5 μL/min) was injected per hemisphere into the amygdala; the injectors were left for another minute to allow diffusion into the tissue. All drugs were dissolved in saline solution (0.9% wt/vol). Experimental groups were separated according to drugs infusions: saline solution (SS, $n = 9$); DL-2-amino-5-phosphonovaleric acid (APV 10 mg/mL, $n = 8$) (Tocris Bioscience); or 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate (CNQX 1mg/mL, $n = 9$) (Sigma-Aldrich). Twenty minutes after micro-infusion, all groups had access to 30 mL of a 0.1% (wt/vol) sodium saccharin for CTA memory retrieval during 15 min. Saccharin consumption over CTA retrieval was measured and microdialysis was carried out until 10 fractions were collected. Saccharin intake during CTA retrieval is reported as percentage of consumption of saccharin during the CTA acquisition (% Acquisition consumption = saccharin solution intake during retrieval × 100/mean saccharin intake during acquisition). Microdialysate samples were analyzed as described above.

Pharmacological disruption of the CTA behavioral response in the IC

Bilateral steel cannulas (9 mm length and 23 gauge) were implanted using standard stereotaxic protocols targeting the IC (AP + 1.2 mm; L +5.5 mm; DV −3 mm). The behavioral scheme was performed as described above. During CTA acquisition all animals were exposed during 15 min to 30 mL of SAC in a graded bottle and 15 min afterward they received an i.p. LiCl injection (0.2 M, 7.5 mL/kg). CTA retrieval was carried out 72 h after acquisition. Rats were divided into five groups counterbalanced according to their acquisition mean consumption. Before retrieval, an injector was inserted into each guide cannula aiming to the IC extending 2 mm below the tip, injection needles were connected via polyethylene tubing into 10 μL Hamilton syringes, driven by an automated microinfusion pump (Carnegie Medicine). A volume of 1 μL (0.5 μL/min) was infused per hemisphere into the IC; the injectors were left for another minute to allow diffusion into the tissue. All drugs were dissolved in saline solution (0.9% wt/vol). Experimental groups were separated accordingly to drugs infusions: saline solution (SS, $n = 6$); DL-2-amino-5-phosphonovaleric acid (APV 10 mg/mL, $n = 6$) (Tocris Bioscience); 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate (CNQX 1mg/mL, $n = 7$) (Sigma-Aldrich); Propanolol (PROP 5 mg/mL, $n = 7$) (Sigma-Aldrich); or SCH-23390 (SCH 2 mg/mL, $n = 6$) (Sigma-Aldrich). Twenty minutes after micro-infusion, all groups had access to 30 mL of a 0.1% (wt/vol) sodium saccharin for CTA memory retrieval during 15 min. Saccharin consumption over CTA retrieval was measured. Saccharin intake during CTA retrieval

is reported as a percentage of consumption of saccharin during the CTA acquisition (percentage acquisition consumption = saccharin solution intake during retrieval × 100/mean saccharin intake during acquisition).

Histology

Cannulae placement was verified at the end of behavioral procedures, animals were sacrificed with an overdose of sodium pentobarbital and transcardially perfused with physiological saline solution. Brains were removed and stored in paraformaldehyde (4% solution in phosphate buffered saline) for 24 h. After a sucrose gradient treatment, coronal sections of 40 m were cut and stained with cresyl violet. Samples were then examined under a light microscope to corroborate the correct placement of cannulas.

Statistics

Statistical analysis of the changes in the extracellular levels of neurotransmitters within IC was done using repeated-measures ANOVA or Two-way ANOVA where appropriate with Bonferroni's post hoc test. A nonpaired Student's *t*-test was used to determine aversion on the CTA test. To evaluate the correlation between extracellular levels of neurotransmitters within the IC and the consumption of saccharin, a two-tailed Spearman correlation coefficient was calculated. A multi-factorial ANOVA with Bonferroni's post hoc test were used to analyze changes in the extracellular levels of neurotransmitters within IC during pharmacological manipulations of the amygdala upon CTA retrieval. Saccharin consumption between groups during retrieval was analyzed with one-way ANOVA with Fishers LSD post hoc test. The value of $P < 0.05$ was considered statistically significant. Prism 6 (GraphPad software) was used to perform analysis and plots.

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