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A Role for the Insular Cortex in Long-Term Memory for Context-Evoked Drug Craving in Rats

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Drug craving critically depends on the function of the interoceptive insular cortex, and may be triggered by contextual cues. However, the role of the insula in the long-term memory linking context with drug craving remains unknown. Such a memory trace probably resides in some neocortical region, much like other declarative memories. Studies in humans and rats suggest that the insula may include such a region. Rats chronically implanted with bilateral injection cannulae into the high-order rostral agranular insular cortex (RAIC) or the primary interoceptive posterior insula (pIC) were conditioned to prefer the initially aversive compartment of a 2-compartment place preference apparatus by repeatedly pairing it to amphetamine. We found a reversible but long-lasting loss (ca. 24 days) of amphetamine-conditioned place preference (CPP) and a decreased expression in the insula of zif268, a crucial protein in memory reconsolidation, when anisomycin (ANI) was microinjected into the RAIC immediately after the reactivation of the conditioned amphetamine/context memory. ANI infusion into the RAIC without reactivation did not change CPP, whereas ANI infusion into pIC plus caused a 15 days loss of CPP. We also found a 24 days loss of CPP when we reversibly inactivated pIC during extinction trials. We interpret these findings as evidence that the insular cortex, including the RAIC, is involved in a context/drug effect association. These results add a drug-related memory function to the insular cortex to the previously found role of the pIC in the perception of craving or malaise.
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INTRODUCTION

Relapse into drug abuse is one of the cardinal signs of addiction, and abstinent drug addicts may relapse into consumption when they feel a hard-to-resist craving for the drug, even after a long drug-free period. The risk of drug craving increases with stress, drug intake, or learned cues such as drug-related context (O'Brien, 1997; Volkow and Li, 2005). Drug craving critically depends on the function of the insula (Contreras *et al*, 2007; Naqvi *et al*, 2007; Hollander *et al*, 2008; Forget *et al*, 2010; Abdolahi *et al*, 2010; Scott and Hiroi, 2011), a key cortical region for interoception, that is, the perception of bodily states, emotions, and needs (Craig, 2004; Damasio *et al*, 2000), such as the need for drug in addicts. The primary interoceptive posterior insula (pIC) of rats is considered a primary sensory area that receives viscerosensory (Cechetto and Saper, 1987) and nociceptive nonspecific (Gauriau and Bernard, 2004) thalamic inputs; pIC is reciprocally connected to the rostral agranular insular cortex (RAIC) through intermediate insular relays (Shi and Cassell, 1998; Kimura *et al*, 2010), whereas the RAIC may be considered a high-order interoceptive cortex (see below and Discussion). Context-evoked drug craving implies the learning of an association between a place and the effects of a specific drug; however, it is not known whether the insula stores such an association or where, among the basic subdivisions of the insular cortex, that association might take place. We hypothesized that the RAIC rather than the pIC may be involved in such an association because the former has the requisite associative connections with regions representing spatial context (Insausti *et al*, 1997; Kerr *et al*, 2007) and has direct connections to premotor regions in the frontal cortex and amygdala (Shi and Cassell, 1998). Moreover, high order, but not primary sensory cortices are able to store remote memories of sensory stimuli made relevant through emotional experience (Sacco and Sacchetti, 2010). We used conditioned place preference (CPP) to assess drug-related context learning, and compared the effect of an amnesic intervention into the RAIC *vs* the pIC (experiment 1). The amnesic intervention consisted of locally injecting a protein synthesis inhibitor or an NMDA receptor blocker (Nader *et al*, 2000; Sara, 2000) shortly after retrieval of the

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place-drug memory, taking advantage of the fragility of consolidated memories shortly after being retrieved. We complemented this approach (experiment 2) by testing whether we could accelerate the extinction of context-induced craving by repeatedly pairing the context previously associated with amphetamine with the absence of craving induced by reversibly inactivating pIC with a voltage-dependent sodium channel blocker (Contreras *et al*, 2007). Facilitated extinction has been tested in exposure therapy (Rothbaum and Davis, 2003), involving the experience of trauma-related cues in the absence of danger, and therefore with reduced emotion-related interoceptive cues.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats from the Faculty of Biological Sciences Animal Care Facility, weighing 293–310 g, were used. Rats individually housed had permanent access to water and food pellets. The experimental protocols included controlled environmental temperature at 22 °C, and were carried out in accordance to the NIH (USA) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). The institutional Bio Safety and Ethical Committee approved these protocols, aimed to minimize the number of rats used and their suffering.

Surgical Procedures

Rats were anesthetized with an intraperitoneal injection of 100 mg/kg of ketamine (Imalgene; Rhodia Merieux) plus 20 mg/kg of xylazine (Rompun; Bayer), and placed in a stereotaxic apparatus to implant sterile stainless-steel guide cannulae (4.5 mm long, 26 G; Plastics One, Roanoke, VA) fixed to the skull with screws (Plastics One) and dental acrylic, and sealed with an occluder. The injection cannulae (33 G; Plastics One) protruded 2 mm from the tip of the guide cannulae. The injection cannulae were aimed at the following coordinates of the Swanson's atlas (Swanson, 1998): pIC (VISC in Swanson's nomenclature), bregma −0.51 mm; midline 5.0 mm; and depth from the cranial surface 6.5 mm. RAIC (AI in Swanson's nomenclature), bregma +2.8 mm; midline 4.0 mm; and depth from the cranial surface 6.5 mm. The placement was verified by analyzing the location of the tip of the cannulae in Nissl-stained sections (Figure 1 and Supplementary Figure S1). The guide cannulae for the pIC were implanted in divergent direction at 10 °C from the vertical. Antibiotics (Enrofloxacin 5%; 19 mg/kg intraperitoneally; Bayer) and an anti-inflammatory (Ketophen; 0.2 mg/kg intraperitoneally; Rhodia Merieux) were administered at the end of surgery. Rats were allowed to recover for a week.

Cortical injections. The injection cannulae were coupled to a 10 μl Hamilton syringe by a polyethylene tubing (inner diameter 1.27 mm; Plastics One) filled with the protein synthesis inhibitor anisomycin (ANI, 100 μg/1 μl per side; Sigma-Aldrich), or with the competitive NMDAR antagonist D(−)-2-amino-5-phosphonopentanoic acid (AP5; 5 μg/1 μl per side; Sigma-Aldrich) or with 1 μl of 0.75% bupivacaine, (Laboratorios Sanderson, Santiago, Chile) or sterile saline; and inserted into the guide cannula after removing the occluder. Injections were made immediately after a test session and during 1 min on each side; the injection needle was left in place for 2 min, and then slowly removed and the occluders reinserted. The ANI dose we used produces >90% protein synthesis inhibition lasting >90 min (Rosenblum *et al*, 1993).

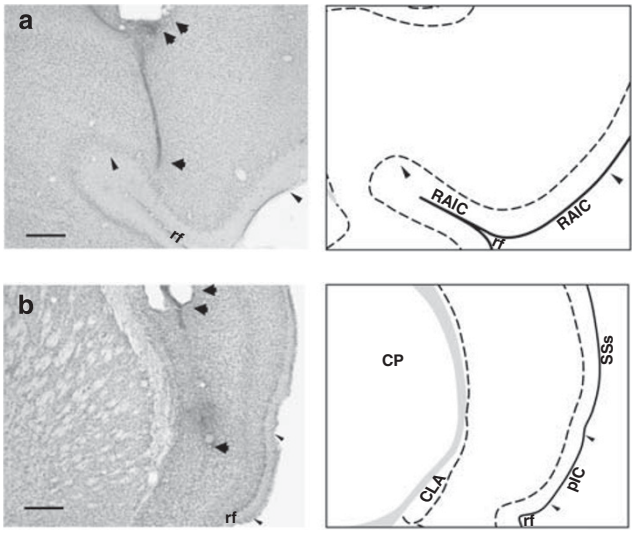

Figure 1 Representative examples of injection sites in the rostral agranular insular cortex (RAIC) (a; indicated by arrowheads) and in the primary interoceptive posterior insula (pIC) (b; indicated by arrowheads). Left panels show photomicrographs of Nissl-stained coronal sections, where the tips of the guide cannulae are indicated by double arrows and the tip of the injection cannulae by single arrows. The location of the injection sites in the different experimental groups is shown in detail in Supplementary Figure S1. Scale bars = 200 μm. CD, caudate–putamen; CLA, claustrum; rf, rhinal fissure; SSs, secondary somatosensory cortex.

Amphetamine-CPP Procedures

We used a place preference apparatus consisting of a dark brown central alley (40 cm long, 30 cm height, and 25 cm wide) connecting a white compartment (45 cm in all dimensions) to a black compartment of the same dimensions. The rats were placed at the center of the alley and video recorded with a zenithal digital camera for 5 or 10 min during the place preference test (PPT) sessions, or for 30 min when studying zif268 expression. The latter groups were left for an additional 30 min in their home cages before perfusion fixation. A week after surgery (day 8, see Figure 2), the rats were habituated to the place preference apparatus for 10 min, and the time spent in each room was measured (PPT1). Next day D-amphetamine (a kind donation of Laboratorios Chile S.A., Santiago, Chile; 1.5 mg/kg dissolved in 1 ml of sterile saline) or 1 ml saline (saline controls) were injected intraperitoneally The rats were immediately left in the white room for 30 min and then returned to their home cages. On alternate days amphetamine-experienced and saline control rats were injected with saline and left for 30 min in the black room. Each rat received 10 injections in successive days (Figure 2a). The second PPT took place on

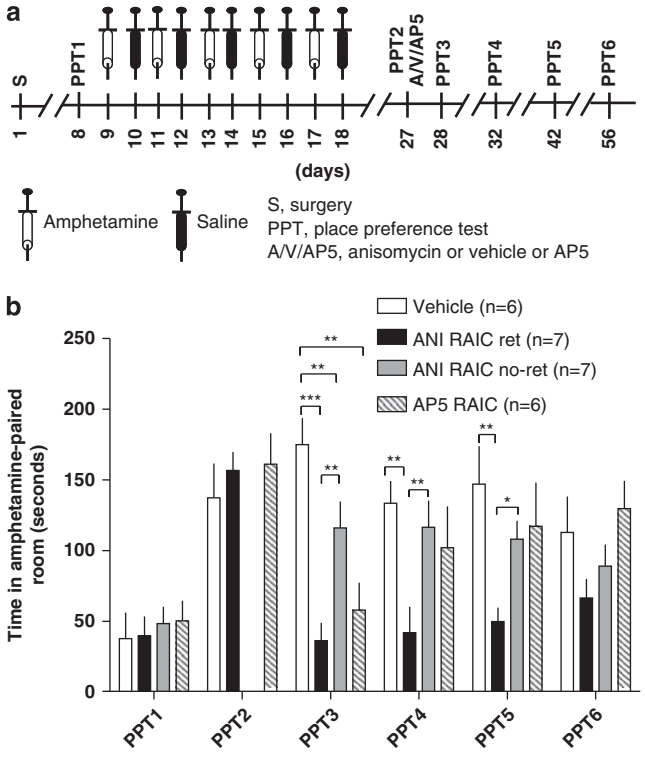

Figure 2 Bilateral microinjection of anisomycin (ANI) into the rostral agranular insular cortex (RAIC) caused a medium term loss of conditioned place preference (CPP) in amphetamine-experienced rats. (a) Timeline of the protocol used in these experiments. Rats were conditioned to prefer the white (and naturally aversive) compartment of a place preference apparatus. During the test sessions (place preference test (PPT)) the rats were placed for 5 min in this apparatus, and the time spent in each compartment was recorded. (b) A single bilateral ANI microinjection into the RAIC on day 27 (A/V), immediately after retrieval of the context–drug memory (ANI RAIC ret group, PPT2) resulted in the loss of the CPP up to PPT6, that is, 39 days after the last drug–context pairing. In contrast, the vehicle group showed a persistence of this memory for at least 39 days after the last conditioning (see text). ANI without retrieval and AP5 plus retrieval decreased the CPP for the amphetamine-paired compartment only at PPT3 (see text). Two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison method. $*p<0.05$; $**p<0.01$; $***p<0.001$. Error bars indicate SEM; *n* is the number of rats.

day 27, that is, 10 days after the last amphetamine (or saline) administration.

Histology. The animals were deeply anesthetized with 7% chloral hydrate (350 mg/kg intraperitoneally) and perfused through the left ventricle with a saline flush (100 ml), followed by 500 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). The brains were post-fixed in the same fixative for 2 h, and transferred to 30% sucrose with 0.02% sodium azide in PBS until they sank. Brains were cut frozen under dry ice in the coronal plane, at 50 μm thickness, using a sliding microtome. We obtained three alternate series of sections from each brain. One series was stained with cresyl violet and another was used for immunohistochemistry.

Immunohistochemistry. Free-floating sections were incubated in 0.3% H_2O_2 in PBS for 30 min, rinsed in PBS, and transferred to the blocking (0.4% Triton X-100, 0.02% sodium azide, 3% normal goat serum in PBS) solution for 1 h. The sections were then transferred to the primary antibody incubation solution, and left there overnight at room temperature. This incubation solution contained the zif268 polyclonal antibody (sc-110, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1 : 2500 in the blocking solution. The sections were rinsed in PBS for 1 h before being incubated in the secondary antibody solution (Biotin-SP-conjugated AffiniPure goat anti-rabbit IgG (H + L) from Jackson ImmunoResearch, PA; diluted 1 : 1000 in 0.4% Triton X-100, 1.5% normal goat serum in PBS). After rinsing for 40 min, the sections were incubated for 1 h in Vectastain ABC Elite kit (Vector Laboratories, CA), diluted 1 : 500 in PBS, rinsed, and incubated in a 0.05% 3–3′ diaminobenzidine hydrochloride solution containing 0.003% H_2O_2 and 0.05% nickel chloride to get a dark blue reaction product. The specificity of the zif268 antibody we used was tested by the manufacturer.

Cell counting. The number of zif268-ir neurons was determined in coronal sections through the insular cortex with the help of a camera lucida and a Nikon microscope fitted with an $\times 10$ objective. For the RAIC, we sampled 2 sections per rat, from bregma +4.85 to +3.60, and used a $0.25 \times 1\,mm^2$ counting grid; from bregma +2.80 we used a $0.5 \times 1.25\,mm^2$ counting grid and sampled 1 section per rat; and from bregma +1.70 to +1.20 we sampled 2 sections per rat and used a $0.5 \times 1\,mm^2$ counting grid. For pIC we sampled 4 sections per rat, from bregma 0.95 to −0.26, and used a $0.25 \times 1\,mm^2$, and finally we sampled 4 sections per rat, from bregma −0.51 to −2.45 mm with a $0.5 \times 1\,mm^2$ counting grid. For the primary somatosensory cortex, we sampled 4 sections per rat, from bregma −0.82 to −1.78 mm with a $0.5 \times 1\,mm^2$ counting grid.

Experimental Design

Experiment 1: Effect of amnesic intervention in RAIC vs *pIC in the expression of amphetamine CPP.* On day 27 and immediately after PPT2 (retrieval group; Figure 2a), amphetamine-experienced rats were injected into the insular cortex with ANI (either RAIC or pIC), AP5 (into RAIC only), or sterile saline; 24 h later they were tested in the place preference apparatus (PPT3) for 5 min. On the subsequent 5 (PPT4), 15 (PPT5), and 29 (PPT6) days after the injections into the insular cortex, the rats were retested during 5 min. Another group of amphetamine-conditioned rats received bilateral ANI injection into the RAIC and left at their home cages, that is, without re-exposure to the conditioning context and served as a no retrieval control.

To rule out a possible aversive effect of ANI when injected into the RAIC that might explain the loss of CCP after PPT2, we tested a group of six drug-naïve rats. They were bilaterally injected with ANI into the RAIC and placed in the dark compartment a day after testing for their place preference, and retested the day after the injection.

Experiment 2: Effect of pIC inactivation during extinction trials on CPP extinction. On the third day after PPT2, amphetamine-experienced rats were subjected to an extinction protocol for 5 min per session after a bilateral injection of bupivacaine or sterile saline into the pIC. An extinction

session (one per day) consisted of leaving the rats in the place preference apparatus during 10 min, allowing them to freely access both chambers. Bupivacaine reversibly inactivates peripheral nerves for 30–60 min at the dose we used (Farnham and Pilowsky, 2009). At 24 h after the last extinction session, the rats were tested again for place preference and half of them were prepared for histology afterwards. The place preference of the other half was tested 10 days later to check for memory loss in a longer time period.

Statistics. We used the GraphPad Prism 5 software. The number of zif268-ir neurons in the insular cortices was analyzed using the Student's *t*-test. The time spent in amphetamine-paired room was analyzed with one- or two-way ANOVA, followed by Bonferroni multiple comparisons method.

RESULTS

Rats were conditioned to prefer the white compartment of a two-compartment biased apparatus, over-riding the natural preference for the black compartment, by repeatedly pairing amphetamine administration with the aversive (white) compartment, while the black compartment was paired with saline administration (Figure 2a). In all, 52% of the amphetamine-treated rats showed a change in place preference (2 SD from the average preference for the aversive compartment of saline-treated rats, that is, $>1.08 \pm 0.61$ min), and these rats proceeded to the rest of the protocol; only data from these rats were analyzed, and the rats that did not condition were not included in the PPT1 groups.

Experiment 1: Effect of Amnesic Intervention in RAIC *vs* pIC in the Long-Term Expression of Amphetamine CPP

Drug-naïve rats spent little time in the white compartment during the first test (PPT1), conducted before conditioning. At 10 days after the last amphetamine administration (see timeline in Figure 2a), all groups showed a significant increase in the preference for the amphetamine-paired compartment (two-way ANOVA, PPT (time) factor: $F_{(1,\ 56)} = 29.64$, $p < 0.0001$). The time spent in the black compartment (not paired with amphetamine) decreased from PPT1 to PPT2 (Supplementary Figure S2). Amphetamine-conditioned rats injected with vehicle into the RAIC showed a significant and long-lasting CPP for the amphetamine-paired compartment at all PPTs: (one-way ANOVA, Bonferroni post-test; $F = 8.6$, $p < 0.0001$), that is, even after 38 days of abstinence.

ANI was successfully microinjected into the RAIC (both sides; Figure 1 and Supplementary Figure S1) in 14 rats (Figure 2b), on day 27. Seven of these rats received ANI immediately after PPT2 (Figure 2b, black columns), while the other seven rats received ANI without previous PPT2 (ie, without retrieval). When tested the next day (PPT3 in Figure 2b), the animals treated with ANI and retrieval reverted to the normal avoidance of the white compartment and spent as little time in this compartment as drug-naïve rats (ie, all rats at PPT1). The same loss of CPP was observed in all subsequent tests, except for the last one, which was run on day 56 of the protocol, that is, 39 days after the last amphetamine administration: treatment ($F_{(1,\ 66)} = 32.92$, $p < 0.0001$), time [$F_{(5,\ 66)} = 7.73$, $p < 0.0001$), and interaction ($F_{(5,66)} = 6.02$, $p = 0.001$) had a significant effect. Bonferroni post-test indicated that at all PPTs, except for PPT6, these rats spent significantly shorter time in the amphetamine-paired compartment than the rats treated with vehicle into the RAIC plus retrieval (Figure 2b, white columns). In the case of ANI into the RAIC without retrieval, a significant effect of treatment ($F_{(1,\ 55)} = 4.79$, $p = 0.0329$) and time ($F_{(4,\ 55)} = 9.293$, $p < 0.0001$), but not interaction, was observed (Figure 2b, gray columns). The decrease in CPP of ANI no-retrieval compared with vehicle showed no significance effect after post-test (Bonferroni). We interpret this ANI effect as nonspecific, given the significant differences with ANI plus retrieval at PPT3, PPT4, and PPT5.

To test for the relevance of using ANI *vs* another classical way to interfere with memory reconsolidation (Tronson and Taylor, 2007; Sara, 2010), we injected the NMDA receptor blocker D-AP5 into the RAIC immediately after PPT2 (Figure 2b, hatched columns). We found that this intervention caused a short-term loss of CPP detected only at PPT3: time (PPT) ($F_{(5,\ 60)} = 5.10$, $p = 0.0006$) and interaction ($F_{(5,\ 60)} = 2.73$, $p = 0.027$) had an effect but not treatment ($p = 0.116$).

To test if the loss of CPP expression up to PPT5 was specific to the region of the insula treated with ANI—as predicted by the assumption that, in the rat, RAIC is a higher-order interoceptive cortex relative to the pIC—we repeated the ANI plus retrieval experiment, but this time we microinjected ANI into the pIC on both sides, immediately after retrieval (Figure 3). This is the same insular cortex region where we temporarily prevented drug craving by reversible inactivation with bilateral lidocaine (Contreras *et al*, 2007). We found in this case a temporary loss of amphetamine-CPP that recovered in 15 to 25 days after the last context–drug conditioning. Treatment ($F_{(1,\ 60)} = 18.12$, $p < 0.001$) and time (PPT) ($F_{(5,\ 60)} = 5.35$, $p = 0.0004$) had

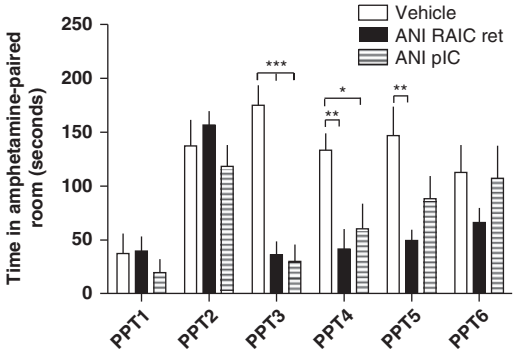

Figure 3 Transient suppression of amphetamine-conditioned place preference (CPP) by anisomycin (ANI) plus retrieval in two different regions of the insular cortex. Timeline of the protocols as in Figure 2a; place preference tests took 5 min. Bilateral microinjection of ANI in the primary interoceptive posterior insula (pIC) after retrieval (at place preference test 2 (PPT2)) suppressed CPP at PPT3 and PPT4, that is, for less than a week. Vehicle controls and ANI-ret groups are the same as that shown in Figure 2b. Two-way analysis of variance (ANOVA) followed by Bonferroni post-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

effect, and the post-test revealed significant differences at PPT3 and PPT4. When comparing the effect of ANI into RAIC or pIC plus retrieval, we found a significant effect of time (PPT) ($F_{(5, 66)} = 10.77$, $p < 0.0001$), but not of treatment ($p = 0.574$) or interaction ($p = 0.139$).

To control for a possible aversive effect of ANI that might explain the change in CPP after PPT2, we made bilateral ANI infusion into the RAIC of drug-naïve rats, while they were in the black compartment and found no change in their preference for the black compartment (Supplementary Figure S3), indicating that ANI into the RAIC *per se* did not change the preference for the compartment paired with ANI.

We counted neurons immunoreactive to zif268, a memory reconsolidation-related protein (Hall *et al*, 2001), in the RAIC, in the pIC and in the primary somatosensory cortex (S1), of rats killed 60 min after the last PPT. We found (Figure 4) that ANI/retrieval treatment on the RAIC decreased zif268-ir in both insular cortices (see legend of Figure 4 for statistical details), compared with the rats that received ANI uncoupled to retrieval and showed no loss of CPP. No significant change was found in S1, used as a control cortex. In three rats injected with vehicle into the RAIC plus retrieval, we found values similar to those of the ANI plus retrieval group (RAIC, 435.7 ± 199.8 zif268-ir neurons per mm^2; pIC, 623.3 ± 154.9; S1, 107.3 ± 34.51), but this small sample did not allow for statistical comparisons. The lack of saline plus retrieval or saline plus no-retrieval controls prevented to establish a stronger relation between zif268 expression and the behavioral results.

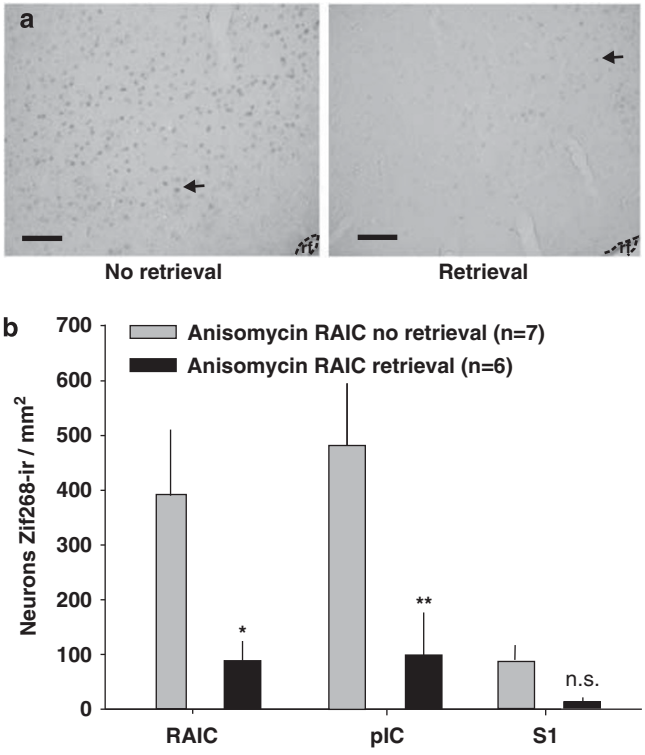

Figure 4 Anisomycin (ANI) administration after retrieval (place preference test 2 (PPT2) in Figure 2) decreased the expression of the zif268 protein in the rostral agranular insular cortex (RAIC) and in the primary interoceptive posterior insula (pIC), but not the primary somatosensory cortex (S1). (a) Photomicrographs showing high zif268 immunoreactivity in the RAIC of a rat treated with ANI but without context retrieval (left panel), and a low zif268 immunoreactivity in a rat where ANI was given right after retrieval. (b) Quantification of zif268 expression in three cortical regions. One-tailed Mann–Whitney test. $^*P = 0.037$; $^{**}P = 0.006$; NS, nonsignificant. Error bars indicate SEM; *n* is the number of rats. Scale bars = 100 μm.

Experiment 2: Effect of pIC Inactivation During Retrieval on CPP Extinction

A transient inactivation of pIC causes a temporary loss of drug craving and prevents the expression of lithium-induced malaise (Contreras *et al*, 2007). We interpreted those findings as evidence that these rats neglected the interoceptive signals from their bodies during pIC inactivation. Here we tested whether repeatedly pairing a state of interoceptive neglect with the context previously associated with amphetamine would accelerate the extinction of CPP, because context now signals no craving. This idea came from the rationale for exposure therapy, where accelerated extinction results from exposure to the CS in a less emotional scenario (Rothbaum and Davis, 2003). Extinction is usually characterized by the formation of new associations that compete with old memory traces for behavioral expression (Maren, 2011). In our case, conditioned rats would learn now that the white compartment is associated with the absence of drug craving. Rats were injected bilaterally with bupivacaine or vehicle into the pIC immediately before (Figure 5a) the extinction trials, which consisted of leaving the rats in the place preference apparatus for 10 min. Conditioned rats injected with bupivacaine into pIC reverted to the initial preference for the black compartment during the extinction trials, as exemplified in Figure 5b; one-way repeated-measures ANOVA was followed by Student–Newman–Keuls multiple comparison method; $^*p < 0.01$. This result confirmed our previous findings with lidocaine into pIC (Contreras *et al*, 2007).

Rats treated with vehicle ($n = 10$) did not show extinction of the CPP at any time (Figure 5c). In contrast, rats treated with bupivacaine ($n = 10$ up to PPT3; $n = 5$ at PPT4; see Materials and Methods) showed a loss of CPP expression; treatment ($F_{(1, 60)} = 18.12$, $p < 0.0001$); time (PPT) ($F_{(5, 60)} = 5.35$, $p = 0.0004$); and treatment × time had a significant effect ($F_{(5, 60)} = 2.89$, $p = 0.021$). At PPT3 and PPT4, these rats showed a loss of CPP compared with the vehicle group (Bonferroni post-test).

DISCUSSION

We found a long-lasting, but not permanent loss of amphetamine-CPP when ANI was microinfused into the RAIC immediately after the reactivation of amphetamine/context association. These rats had decreased expression in the insula of zif268, a protein involved in memory reconsolidation. No memory loss was produced when ANI was infused into the RAIC, but without memory reactivation. We found a brief loss of CPP when an NMDA receptor blocker was infused into the RAIC after memory reactivation or when ANI was injected into the pIC after memory reactivation. We also found a loss of CPP that lasted at least

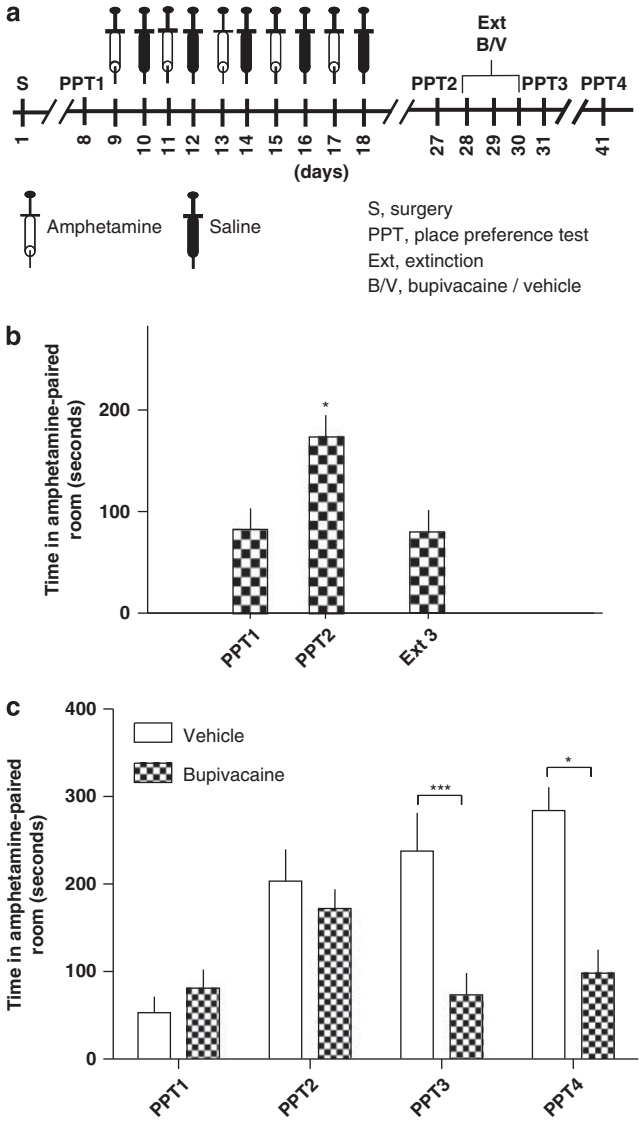

Figure 5 Enhanced extinction of conditioned place preference (CPP) was induced by inactivation of the primary interoceptive posterior insula (pIC) with bupivacaine during successive extinction trials. (a) Timeline. (b) Each bupivacaine injection into the pIC reverted CPP to that shown at place preference test 1 (PPT1). (c) The repeated pairing of extinction trials with reversible inactivation of pIC caused a significant decrease in the expression of CPP for at least 24 days after the last amphetamine administration. The strength of the memory trace is reflected by its persistence for at least 24 days in the vehicle group. Two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison method. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. Error bars indicate SEM; the number of rats per group was 5 for PPT4 and 10 for the rest (see Materials and Methods).

24 days after the last conditioning, induced by inactivating the primary interoceptive insula during extinction. These findings contribute to place the insula as a player in drug craving induced by context, in addition to its role in relapse in abstinent humans (Paulus *et al*, 2005) and in decision-making based on emotions (Sanfey *et al*, 2003).

Underlying a complex motivational disorder such as drug addiction, there are a number of plastic changes involving a variety of brain structures and learning mechanisms (Robbins *et al*, 2008; Kasanetz *et al*, 2010). The present results indicate that the insular cortex, including its RAIC subdivision, is important in the expression of a long-term memory associating drug craving with context. Studies using interference with memory reconsolidation have also shown that the hippocampus, the basolateral amygdale, and the nucleus accumbens (Milekic *et al*, 2006; Li *et al*, 2010; Taubenfeld *et al*, 2010) are also important for the expression of drug-CPP and that interference with reconsolidation in those structures caused amnesia for the CPP that lasts weeks after the last drug-induced place conditioning. However, a neocortical region, such as the insular cortex, may be a better candidate as a hub that links context and drug effect. It is widely accepted that the neocortex (Squire, 2009; Maviel *et al*, 2004), and not the hippocampus, is the region where declarative memories are stored. Similarly, current views on amygdala functions do not include long-term memory storage as one of them (Davis and Whalen, 2001; Pessoa and Adolphs, 2010). Nevertheless, the importance of these structures relative to the RAIC as a site for context–drug effect association remains to be investigated.

Contextual cues, recalled for instance from the place where drug is habitually obtained, are powerful inducers of craving for drug, as they bring into consciousness the bodily sensations and feelings (Damasio *et al*, 2000) linked to the drug—either memories of the pleasurable state induced by the drug or the relief the drug afforded from an unpleasant state. Our results concur with studies indicating that the rat insular cortex stores memory traces of the illness that resulted from ingesting some taste-identifiable food in the conditioned taste aversion paradigm (BermudezRattoni, 2004).

We showed that ANI/retrieval treatment on either RAIC or the pIC caused a long but reversible loss of CPP. Although this amnesic effect was longer when involved with the RAIC rather than pIC, we cannot at present dissect out the relative contributions of these two insular regions to the CPP because there is a possibility that the ANI solution diffused from one insular region to the other (Wanisch and Wotjak, 2008). However, the neural connections of the RAIC fit better into the description of a high-order cortex that might associate context with drug effect: The RAIC receives thalamic input from the medial subdivision of the mediodorsal thalamic nucleus (Krettek and Price, 1977; Allen *et al*, 1991), considered a high-order thalamic nucleus (Groenewegen and Witter, 2004), and from thalamic nuclei that are thought to convey the affective components of nociception (Jasmin *et al*, 2004). Interoceptive information may reach the RAIC from an intermediate, polysensory region of the granular insular cortex located rostral to the primary interoceptive cortex (Kimura *et al*, 2010) and from the adjacent dysgranular insular cortex (Shi and Cassell, 1998; Gabbott *et al*, 2003). Contextual information probably reaches the RAIC from brain regions representing spatial information or context, such as the entorhinal cortex (Insausti *et al*, 1997), closely interconnected with the hippocampus, and the posterior parietal cortex (Kerr *et al*, 2007). In turn, RAIC sends projections to medial prefrontal cortex (Shi and Cassell, 1998), another defining trait for a high-order cortex, which might influence on decision making, such as the decision to spend time in the amphetamine-paired white compartment in spite of the aversion that rats have for bright places. The first-order interoceptive cortex

located in the pIC (Cechetto and Saper, 1987; Ito, 1994; Stehberg *et al*, 2001) has no such associative connections and it is not directly connected with prefrontal cortices. In addition to the feedforward connections from the posterior insula to rostral insula, there are feedback connections from the latter to the former (Shi and Cassell, 1998).

In humans, a hierarchical organization of the insula has been postulated, with an anterior high-order interoceptive cortex that is active during emotional processing and a posterior first-order cortex that is activated in response to more basic interoceptive stimulation (Craig, 2004). It is difficult at this time to relate the present findings with those in humans because a detailed correspondence of the human insula with the rat insular cortex is lacking.

ANI interferes with a memory trace principally by its protein inhibitor function (Alberini, 2008); however, its side effects, such as kinase activation or decreased catecholamine accumulation, may impact on neural circuits supporting a recently activated memory (Sara, 2000; Rudy *et al*, 2006). Perhaps, this complex action of local ANI on neural circuits may explain why this agent was more effective than NMDA receptor blockers in causing the memory loss when infused into the RAIC.

The importance of the insula in drug craving (Contreras *et al*, 2007; Naqvi *et al*, 2007; Hollander *et al*, 2008; Forget *et al*, 2010; Abdolahi *et al*, 2010; Scott and Hiroi, 2011) was confirmed during the CPP extinction trials run under reversible inactivation of pIC with bupivacaine (Figure 5b). We report here that the repeated pairing of context with the inactivation of pIC resulted in a protracted loss of CPP, at least up to 24 days after the last conditioning with amphetamine. This finding suggests that conditioning may have induced a plastic change in pIC to increase the representation of the amphetamine effects on interoceptive cortex, and that such representation might have been disrupted by this accelerated extinction procedure or by ANI into the pIC plus retrieval. However, we did not test if bupivacaine without retrieval may have disrupted CPP. Perhaps, this extinction procedure interfered with something akin to perceptual learning, an increased ability to perceive stimuli that became behaviorally relevant by learning, and that involves plastic changes in primary sensory cortices (Xiong *et al*, 2006; Weinberger, 2007; Gilbert *et al*, 2009), or changes in primary motor cortex (Kleim *et al*, 2003). It is conceivable that the increased importance of a drug as an incentive, along with repeated exposure to the drug, may increase the representation of the bodily state associated with the drug in pIC, under the influence of descending axonal projections from rostral insula; the increase in *zif268* expression in pIC (Figure 4b) during reconsolidation may reflect such plastic change. The opposite finding was reported after inactivation of the medial prefrontal cortex (Hsu and Packard, 2008), namely that infusion of bupivacaine in that cortex prevented the extinction of an amphetamine CPP, in line with the role of the medial prefrontal cortex in extinction of other conditionings.

At present, it is not possible to explain the long-term loss of the drug-CPP as a failure in retrieval or as a disruption of storage (Squire, 2006), because we assessed a behavioral outcome and not an underlying memory mechanism. The decreased expression in the insula of zif268 favors the second option.

In conclusion, we found a long-lasting, but not permanent loss of context–drug association when we interfered with the reconsolidation of this mnemonic function by infusing ANI into the RAIC during retrieval and a long-lasting loss of CPP when pIC was inactivated during extinction of the CPP. These results add a drug-related memory function to the insula, and confirmed the previously found role of the pIC in the perception of drug craving.

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DISCLOSURE

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