Behavioural Brain Research 296 (2016) 70–77

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

A role for the interoceptive insular cortex in the consolidation of learned fear

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HIGHLIGHTS

- The interoceptive insular cortex is required for the long term storage of learned fear in rats.
- The interoceptive insular cortex is not required for the expression of fear during the acquisition of learned fear.
- The long term inactivation of the interoceptive insular cortex decreases the expression of learned fear.
- The expression of conditioned fear increases the neural activity of the insular cortex.

ARTICLE INFO

Article history:
Received 5 May 2015
Received in revised form 20 August 2015
Accepted 24 August 2015
Available online 28 August 2015

Keywords:
Conditioned fear
Insula
Insular cortex
Memory
Consolidation

ABSTRACT

A growing body of evidence suggests that learned fear may be related to the function of the interoceptive insular cortex. Using an auditory fear conditioning paradigm in rats, we show that the inactivation of the posterior insular cortex (pIC), the target of the interoceptive thalamus, prior to training produced a marked reduction in fear expression tested 24 h later. Accordingly, post-training anisomycin infused immediately, but not 6 h after, also reduced fear expression tested the following day, supporting a role for the pIC in consolidation of fear memory. The long-term (*ca.* a week) and reversible inactivation of the pIC with the sodium channel blocker neosaxitoxin, immediately after fear memory reactivation induced a progressive decrease in the behavioral expression of conditioned fear. In turn, we observed that fear memory reactivation is accompanied by an enhanced expression of Fos and Zif268, early genes involved in neural activity and plasticity. Taken together these data indicate that the pIC is involved in the regulation of fear memories.

1. Introduction

The insular cortex (IC) has been implicated in a number of cognitive functions including emotion, memory, interoception, attention, etc [23]. A flow of information has been proposed between the posterior and the anterior insular cortices in humans [12]. This process would involve the integration of emotional and cognitive aspects of information in the anterior IC that starts with the collection of sensory information that reaches the posterior IC [12].

Experimental studies in the rat have provided conflicting results regarding the relationship between the IC and the emotion of fear. Christianson et al. [14] showed that a pre-training lesion or the reversible inhibition of a region of the pIC during training, but not during testing [15], prevents the effect of a safety signal on subsequent fear/anxiety-like behavior. Alves et al. [2] found that inactivation of a more rostral IC region after training or before testing (but not before training) attenuated freezing and cardiovascular responses evoked by context. The lesion of the most caudal IC cortex after training [38] blocked fear-potentiated startle. In contrast, no evidence was found for an involvement of this most caudal IC in fear conditioning [7,24]. When comparing these studies, it seemed that one key difference lies on the specific IC region that was inactivated.

The posterior IC of rats (pIC) is somehow coextensive with the anterior parietal granular IC of [39], the recipient of axonal projections from the interoceptive thalamus located in the ventro-

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http://dx.doi.org/10.1016/j.bbr.2015.08.032
0166-4328/

posterior parvicellular thalamic nuclei [39,1,17], and as such the pIC is constantly receiving information about the condition of the body. The pIC sends axonal projections to more anterior IC regions [39], that in turn are connected to the prefrontal cortex, the amygdala and the hippocampal formation. In this context, the pIC appears as an important hub to distribute interoceptive information to the cortical circuits that regulate emotion.

We hypothesized that the inactivation of the pIC should disrupt the flow of information within the IC and give a view of the possible contribution of interoception to a paradigmatic emotion. We decided to study the effect of interfering with this pIC region at different stages of classical auditory fear conditioning. We used reversible inactivation, inhibition of protein synthesis and immunohistochemistry, in order to determine the role of the pIC in the regulation of fear memories in rats.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (300–350 g) were housed individually with food and water ad-libitum. Room temperature was kept constant at 25 °C and the lights set under a 12–12 h light/dark cycle (lights on at 7:00 AM). All procedures were in strict accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996) and were approved by the Bioethics Committee of Pontificia Universidad Católica de Chile.

2.2. Stereotaxic surgery

Prior to surgery, animals were handled 10 min once daily for 3 consecutive days. Animals were anesthetized with 100 mg/kg of ketamine (Imalgene; Rhodia Merieux) plus 20 mg/kg of xylazine (Rompun; Bayer), placed in a stereotaxic apparatus, and implanted bilaterally with stainless-steel microinjection cannulas aimed to the pIC using the following coordinates: Bregma, −0.51 mm, midline, +5.0 mm, and −6.5 mm from the cranial surface, angled 10° from the vertical, according to the Swanson's atlas [41]. For the primary somatosensory cortex (SSp, in Swanson's nomenclature), we used the following coordinates: bregma −0.46 mm, midline 4.5 mm, and depth 1.5 mm. These cannulas were 4.5 mm long for pIC and 1.5 mm for Ssp (26 G; Plastics One, Roanoke, VA). The cannulas were fixed to the skull with dental acrylic and 3 stainless-steel screws (Plastics One). Right after surgery and for the following 3 days, rats were given injections of Enrofloxacin 5% (19 mg/kg i.p., Bayer) and Ketoprofen (0.2 mg/kg i.p. Rhodia Merieux). Rats were allowed to recover for 7 days prior to any experimental procedure.

2.3. Cortical injections

Injection cannulas were coupled to a 10 μL Hamilton syringe by a polyethylene tubing (inner diameter 1.27 mm; Plastics One) filled with muscimol (MUS, 0.5 μg/μL, 0.5 μL/side; Sigma–Aldrich), the voltage-gated sodium channel blocker neosaxitoxin (NSTX; 32.5 μM/1 μL/side; CRM-MRCBiotoxin, Canada), the protein synthesis inhibitor anisomycin (ANI, 100 μg/μL, 0.5 μL/side; Sigma–Aldrich) or with vehicle (sterile saline; SAL, 0.5 μL/side). Microinjections took 1 min on each side. The injection needle was left in place for additional 2 min to allow for diffusion, slowly removed the injection needle; and replaced the occluders back immediately.

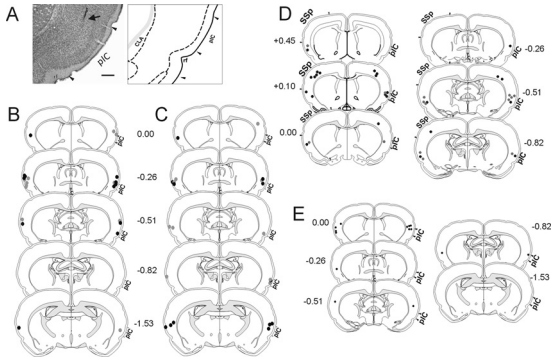

Fig. 1. Representative example of cannula placement in the posterior insular cortex (pIC). A. Left panel, photomicrograph of a Nissl stained coronal section showing the endpoints of the injection cannula tips (arrow) in the pIC, and a schematic drawing (right) modified from Swanson's atlas. B. Muscimol (black circles) or Saline (grey circles) injection sites of Experiment 1. C. Anisomycin (black circles) or Saline (grey circles) injection sites of Experiment 2. D. Neosaxitoxin (black circles) or Saline (grey circles) injection sites of Experiment 3. E. Muscimol (grey circles) injection sites of Experiment 3. Scale bar indicate 500 μm. Abbreviations: CLA, claustrum; rf, rhinal fissure [41].

2.4. Fear conditioning

For the acquisition of auditory fear conditioning, rats were placed in a 27 × 27 × 27 cm conditioning chamber (Harvard Apparatus Model LE1005; context A), equipped with a floor of steel rods to deliver electric footshocks. A different chamber of 64 × 38 × 30 cm (context B), with Plexiglas® floor and situated in a different room, was used to assess conditioning. Prior to the experiments, rats were habituated to both contexts, A and B, by allowing them to explore during 15 min each one for 2 consecutive days. The training (day 1) consisted in presenting 5 habituation tones (CS, 80 dB, 5 KHz, 20 s) followed by a train of 7 tones that co-terminated with a mild footshock (0.7 mA; 0.5 s). The pairings were separated by a variable interval averaging 2 min. Two min after the last CS-US pairing, the rats were taken back to their home cages. On day 2, the rats were placed into the context B, where the CS was presented 15 times with a cadency of one event every 2 min. On day 3, the rats were placed back in the context B to test for retrieval of fear memory by presenting them with two CS. For the long-term inactivation experiment (Experiment 3) the training took longer, two days instead of one, so as to have a stronger conditioning, similar to that modeling post-traumatic stress disorder [37].

2.5. Histology

Once all the procedures were completed, rats were killed with 7% Chloral Hydrate (350 mg/kg i.p.) and perfused transcardially with 500 mL of saline followed by 500 mL of 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). Brains were removed, left in 30% sucrose with 0.02% sodium azide in PBS until they sank, and sectioned frozen under dry ice in the coronal plane, at 50 μm thickness, using a sliding microtome. The sections were stained with Cresyl Violet to verify the cannulas placement (Fig. 1).

2.6. Immunohistochemistry for Zif268 and Fos expression

Coronal brain sections were incubated in 0.3% H_2O_2 in PBS for 30 min, rinsed in PBS and transferred to the blocking solution (0.4% Triton-X100, 0.02% sodium azide, 3% normal goat serum in PBS) for 1 h, and left incubating overnight at room temperature with the primary polyclonal antibody anti-Fos (Ab-5, rabbit polyclonal, from Oncogene, San Diego, CA), diluted 1:20,000, or anti-Zif268 (sc-110, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz,

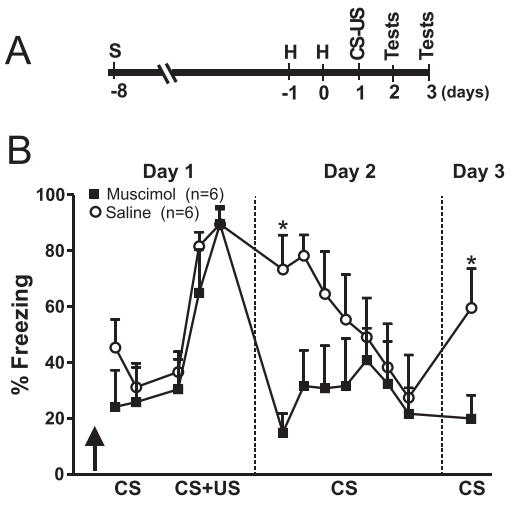

Fig. 2. Inactivation of pIC prior to training impaired retention of conditioned fear. A. Time line of the protocol. B. Freezing time course for muscimol (MUS; $n = 6$) and saline (SAL; $n = 6$) infused rats. MUS infusion into the pIC prior to training (arrow) did not affect the expression of fear during conditioning (Day 1). However, MUS infusion markedly reduced fear expression during extinction trials (Day 2). This difference persisted on day 3. Data are expressed as mean + SEM in blocks of two trials, $^{*}p < 0.05$.

CA) diluted 1:2,000. The following day, sections were rinsed in PBS, and then were incubated in the secondary antibody solution for 1 h (1:1000, Biotin-SP-conjugated goat anti-rabbit IgG, H + L; Jackson Laboratories, West Grove, PA). After rinsing for 40 min, sections were incubated for 1 h in Vectastain ABC Elite Kit (Vector Laboratories, USA), rinsed and incubated in a 0.05% DAB solution containing 0.003% H_2O_2, and 0.05% nickel chloride to obtain a dark blue reaction product.

2.7. Cell counting

The number of Zif268-ir and Fos-ir neurons was determined live in coronal sections with a camera lucida coupled to a Nikon microscope fitted with a 10X objective. For the anterior, higher order subdivision of the IC, the rostral agranular IC (RAIC), we sampled 2 sections per rat from bregma +4.85 to + 3.6 mm, and used a $0.25 \times 1\ mm^2$ counting grid; for bregma +2.80 mm, we sampled 1 section per rat and used a $0.5 \times 1.25\ mm^2$ counting grid; and from bregma +1.70 to +1.20 mm, we sampled 2 sections per rat and used a $0.5 \times 1\ mm^2$ counting grid. For the pIC, from bregma +0.95 to −0.26 mm, we sampled 4 sections per rat, and used a $0.25 \times 1\ mm^2$ counting grid; from bregma −0.51 to −2.45 mm, we sampled 4 sections per rat, and used a $0.5 \times 1\ mm^2$ counting grid. For the SSp, from bregma −0.82 to −1.78 mm, we sampled 4 sections per rat, and used a $0.5 \times 1\ mm^2$ counting grid. For the prelimbic cortex (PL), we sampled 3 sections per rat, from bregma +4,85 to 2,80 mm^2 and used a $0{,}96 \times 1{,}04\ mm^2$ counting grid. Data were analyzed using Two Way ANOVA and Bonferroni post test, or unpaired t-test (one-tailed) when appropriate.

2.8. Experimental design

2.8.1. Experiment 1. Effect of pIC inactivation on conditioned fear retention

The first experiment was aimed to determine whether the pIC has a role in the retention of conditioned fear. The rats were injected bilaterally with MUS or SAL into the pIC 30 min prior to training (arrow in Fig. 2A). The following days (Day 2 and Day 3), the rats were tested for conditioned fear expression in the context B.

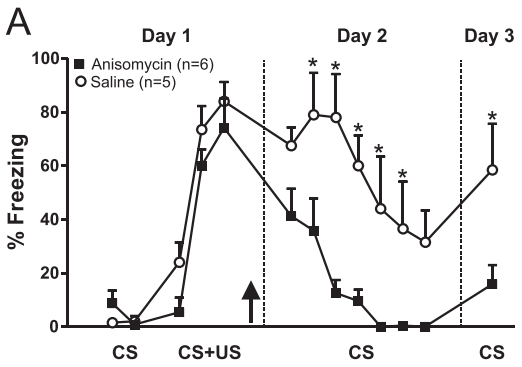

Fig. 3. Infusion of anisomycin (ANI) into the pIC immediately after training impaired the consolidation of conditioned fear. Time course of the freezing response for ANI (black squares; $n = 6$) and saline (SAL; white circles; $n = 5$) infused rats. ANI-infused rats into the pIC right after training (arrow), showed lower levels of freezing than SAL-infused rats during extinction trials (Day 2). This difference persisted on day 3. Data are expressed as mean + SEM in blocks of two trials, $^{*}p < 0.05$.

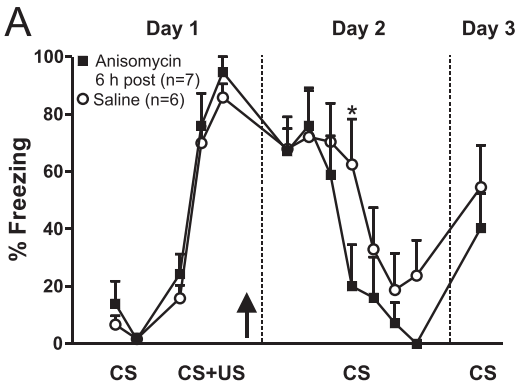

Fig. 4. Infusion of anisomycin (ANI) into the pIC 6 h post training did not impair the retention of conditioned fear. Time course of the freezing response for ANI (black squares; $n = 7$) and saline (SAL; white circles; $n = 6$) infused rats. Rats infused with ANI into the pIC 6 h post-training (arrow) showed similar levels of freezing compared to SAL-infused rats during day 2 and day 3. Data are expressed as mean + SEM in blocks of two trials, $^{*}p < 0.05$.

2.8.2. Experiment 2. Effect of post-training inhibition of protein synthesis in the pIC on consolidation of conditioned fear

It has been described that transient memory representations are transformed into a persisting long-term representation, through a process known as consolidation [20]. This experiment was aimed to test whether consolidation of fear memory take place in the pIC. Immediately following the last CS-US pairing of the training session, rats were infused bilaterally with ANI or SAL into the pIC (arrow in Fig. 3). In a separate group of rats, infusions of ANI or SAL took place 6 h after conditioning (Fig. 4). The following days (Day 2 and Day 3), rats were tested for conditioned fear expression in the context B.

2.8. .3. Experiment 3. Effect of long-term pIC inactivation on repeated reactivations of conditioned fear

Immediately after the first presentation of the CS alone in context B (Test 1, Fig. 5A), the rats received bilateral injections of NSTX or SAL into the pIC, or NSTX into the SSp. On subsequent days, the rats were retested first in the context B and then in the context A during 60 s separated by 30 min. To evaluate whether pIC inactivation by NSTX caused a motor deficit, the locomotor activity was measured during Test 1 (before infusion) and Test 2 (after infusion) by counting the number of crossings over 2 lines that divided context B into 4 equal squares. The freezing before the tone presentation in the context A (pre-tone condition) was used as a measure

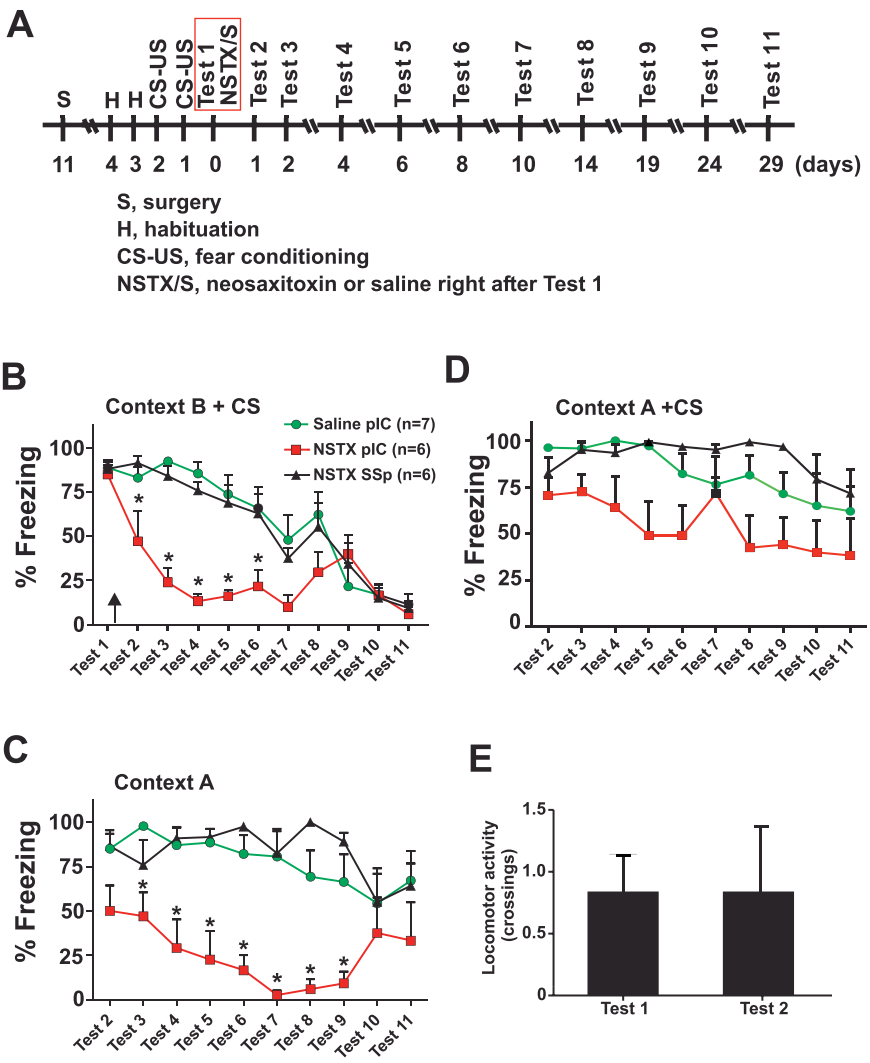

Fig. 5. The long-term inactivation of the pIC caused a prolonged reduction in expression of conditioned fear. A. Timeline of the experimental design. Twenty four hours after fear conditioning in context A, rats were placed in context B and the conditioned freezing was recorded (Test 1). Immediately after tone (CS) presentation, rats were infused with Saline (S) or Neosaxitoxin (NSTX) into the pIC. Another group was infused with NSTX into the primary somatosensory cortex (SSp). In the subsequent days they were returned to both contexts (B and A) and the freezing response was recorded. B. The long-term (*ca.* 7–10 days) inactivation of pIC with a single NSTX infusion reduced the expression of conditioned fear to the auditory CS in the context B. C. The long-lasting inactivation of pIC also reduced the freezing response to the contextual stimulus in the context A. In contrast, the injection of SAL into the pIC or NSTX in the SSp had no effect on the expression of conditioned freezing. D. The infusion of NSTX into the pIC had no effect on the expression of freezing to the tone in the context A. E. The locomotor activity of the rats was not altered by the infusion of NSTX into the pIC. The graph shows the locomotion before (Test 1) and after (Test 2) pIC inactivation. Data are expressed as mean + SEM, $^{*}p < 0.05$, NSTX/pIC vs SAL/pIC and NSTX/SSp.

of freezing to the context in which the fear conditioning occurred. Furthermore, to rule out that the effect of NSTX was due to impaired recall of conditioned fear, we bilaterally inactivated this cortex with MUS, two hours prior to test 3 (see protocol, Fig 6A).

2.8.4. Experiment 4. Expression of early genes accompanying fear memory reactivation

This experiment was aimed to assess the expression of two early genes, c-fos and zif268, known to be involved in synaptic plasticity and used as markers of neural activity [13]. To assess the expression of Fos and Zif268 in the IC, some rats were conditioned for one day, as in Experiment 1, and killed 90 min after one CS presentation in the context B the following day. A control group of rats was spared the foot shocks and exposed only to the CS in the conditioning chamber (unpaired group) and killed 90 min after one CS presentation in context B the following day. We selected this time point to assess Fos and Zif268 because both proteins were at their peaks of expression [13,29].

2.9. Data analysis

Fear expression was assessed as the percentage of time each rat spent in a state of behavioral freezing during tone presentation. For this purpose, behavior was recorded in video by means of a webcam connected to a computer, where videos were stored for off-line analysis by an experimenter blind to treatment. Freezing was defined as the absence of movement, except for breathing, of an attentive rat. In Experiments 1 and 2 the data were plotted as the average freezing of two trials + SEM. The odd number of trials used signifies that the last trial in every stage was excluded from the statistical analysis. Data were analyzed using Two Way ANOVA, with training phase and infusion as the main factors and unpaired *t*-test (two-tailed) when appropriate.

3. Results

The analysis of the Nissl-stained sections revealed that in most cases, the tip of the injection cannula was within the pIC in Experiments 1 and 2 (Fig. 1). The rats with misplaced cannula were

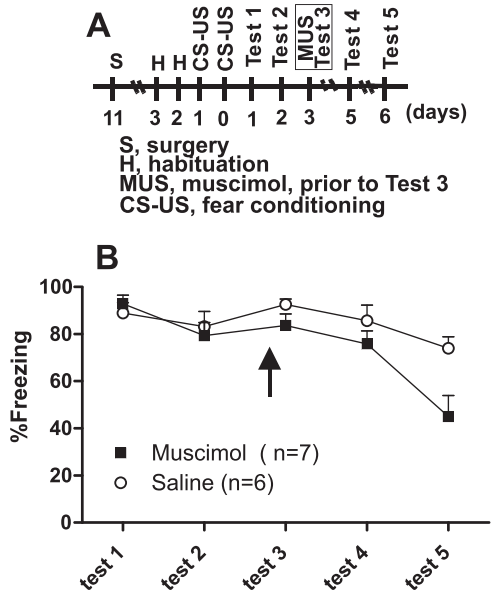

Fig. 6. The inactivation of pIC did not impair recall of conditioned fear. A. Timeline of the experimental design. Each test was conducted in context B. Two hours before test 3, rats were infused with muscimol (MUS) into the pIC. B. Freezing time course for MUS and saline-infused rats. Data are expressed as mean + SEM.

discarded from the behavioral analysis. We paid special attention to signs of cell damage around the injection site of NSTX. The histological examination of the injection sites, 4 weeks after a single administration of NSTX, revealed no significant damage to cortical neurons, reactive gliosis, or inflammatory reaction.

3.1. Experiment 1. Effect of pIC inactivation on conditioned fear retention

On day 1 (Fig. 2B), both, MUS and SAL-infused groups acquired a robust conditioned response to the tone, evidenced as a high level of freezing in the last block (Fig. 2B, SAL 89.6%, MUS 89.6%; $t_{10} = 0.00$, $p > 0.999$). This result indicates that the inactivation of the pIC had no effect in fear expression during conditioning. However, during the extinction trials on the next day (day 2 in Fig. 2B), conditioned rats injected with MUS into the pIC showed a significantly lower level of freezing compared to the SAL-infused group. A Two Way ANOVA revealed a significant interaction between infusion and trial block ($F_{6,60} = 4.207$, $p = 0.0013$), but no main effect of infusion ($F_{1,60} = 2.497$, $p = 0.1451$). The interaction is consistent with a significant difference in freezing between controls and MUS-infused rats on the first block (SAL 73.3%; MUS 15%; $t_{10} = 3.063$, $p < 0.05$). Furthermore, during the test for retrieval of fear memory performed on day 3, MUS-infused rats showed a lower level of freezing than control rats ($t_{10} = 2.503$, $p = 0.0313$). These data suggest that the activity of the pIC is important for fear memory consolidation.

It has been reported that MUS-induced inactivation can last for several hours [44], and that a fear memory needs a few hours after acquisition to consolidate. Therefore, one possible explanation for the effect we observed is that the neural activity of the pIC following training remained low and that this activity is important for long-term storage of a fear memory. In this sense, we hypothesized that synaptic plasticity dependent on protein synthesis was important for long-term storage of fear memory in the pIC. The next experiment was aimed to address this issue.

3.2. Experiment 2. Effect of post-training inhibition of protein synthesis in the pIC on consolidation of conditioned fear

Immediately following fear conditioning, the rats were infused bilaterally with ANI or SAL into the pIC (arrow in Fig. 3). Both groups of rats showed a robust freezing response during conditioning in the last block, with no significant difference between them (Fig. 3A, SAL 84%, ANI 74%; $t_9 = 0.88$; $p > 0.05$). During extinction trials (day 2), a faster decrease in freezing was evidenced in the ANI group as extinction progressed (Fig. 3). A Two Way ANOVA revealed a significant effect of time ($F_{6,54} = 8.955$, $p < 0.0001$) and infusion ($F_{1,54} = 16.16$, $p = 0.003$), but not of interaction ($F_{6,54} = 1.273$, $p = 0.2853$). Post hoc comparisons show a significant decrease in freezing at blocks 2 (SAL 79%, ANI 36%; $t_9 = 2.872$; $p < 0.05$) 3 (SAL 78%, ANI 13%; $t_9 = 4.358$; $p < 0.001$), 4 (SAL 60%, ANI 10%; $t_9 = 3.354$; $p < 0.01$) and 5 (SAL 44%, ANI 0%; $t_9 = 2.928$; $p < 0.05$). Furthermore, during the test for retrieval of fear memory (day 3), ANI-infused rats showed a lower level of freezing than control rats ($t_9 = 2.515$, $p = 0.0331$). In order to rule out that the deficits in retention of fear memory were due to impairing effects of MUS or ANI that lasted beyond the consolidation time window, we performed infusion of ANI 6 h post-training into the pIC. We selected this time point because it has been shown to be an insensitive period to protein synthesis inhibition [6]. Six hours post-training, rats were bilaterally infused with ANI or SAL into the pIC (Fig. 4). Robust conditioned responses were observed during training (day 1), with no significant difference between the groups in the last block (Fig. 4, SAL 85%, ANI 95%; $t_{11} = 0.88$; $p > 0.05$). During the extinction trials (day 2), the rats infused with ANI into the pIC showed similar levels of freezing to that of the SAL group, during the firsts blocks. A Two Way ANOVA revealed a significant effect of time ($F_{6,54} = 17.53$, $p < 0.0001$) and a significant interaction between time and infusion ($F_{6,54} = 2.573$, $p = 0.0289$), but not of infusion ($F_{1,54} = 3.245$, $p = 0.1050$). This interaction was driven by the significant difference in freezing in the fourth block between both groups (Fig. 4, SAL 63%, ANI 20%; $t_{11} = 3.534.$; $p < 0.01$). Retrieval of fear memory (day 3) revealed no difference between both groups ($t_{11} = 0.7929$, $p > 0.05$).

Taken together, these data show that the neural activity and the protein synthesis in the pIC following conditioning are important for the consolidation of auditory conditioned fear memory.

3.3. Experiment 3. Effect of long-term pIC inactivation on repeated reactivations of conditioned fear

This experiment was aimed to test the effect of a protracted (about 7–10 days, see discussion inactivation of the pIC on repeated reactivations of conditioned fear across several days. Immediately after Test 1, rats were infused with NSTX or SAL into the pIC. A third group of rats was infused with NSTX into the SSp. A Two Way ANOVA revealed a significant effect of test day ($F_{10,160} = 24.21$, $p < 0.0001$), infusion ($F_{2,160} = 20.39$, $p < 0.0001$) and interaction ($F_{20,160} = 3.671$, $p < 0.0001$). Twenty-four hours after fear conditioning, all groups showed similar levels of freezing during the first CS presentation in the context B (Fig. 5B, Test 1, SAL/pIC 88.9%, NSTX/pIC 85%, NSTX/SSp 88.2%, p's > 0.05; Bonferroni post test). On the following days, NSTX/pIC rats showed a significant reduction of conditioned freezing in context B relative to SAL/pIC and NSTX/SSp during tests 2–6 (Bonferroni post test, pís < 0.05).

Context-induced freezing (measured before tone presentation) in the context A (the context in which fear was acquired) was also affected by the inactivation of pIC (Fig. 5C). A Two Way ANOVA revealed a significant effect of test day ($F_{9,144} = 2.271$, $p = 0.0208$), infusion ($F_{2,144} = 15.91$, $p = 0.0002$) and of interaction ($F_{18,144} = 2.039$, $p = 0.0111$). NSTX/pIC group showed a significant decrease in freezing compared to SAL/pIC and NSTX/SSp, during tests 3–9(Bonferroni post test, pís < 0.05).

We also measured conditioned freezing when the rats were exposed to both, context and the CS. A Two Way ANOVA revealed a significant effect of test day ($F_{9,144} = 3.481$, $p = 0.0006$) and infusion ($F_{2,144} = 7.665$, $p = 0.0046$), but not for interaction ($F_{18,144} = 0.9384$, $p = 0.5340$). NSTX/pIC rats displayed similar levels of freezing during all tests compared to SAL/pIC rats (Fig. 5D), except for test 5 ($p < 0.05$, Bonferroni post test), whereas significant differences were observed between NSTX/pIC and NSTX/SSp during tests5, 6, 8 and 9 (Bonferroni post test, $p < 0.05$).

We observed no effect of NSTX on general ambulation (Fig. 5E, $p = 0.3649$), or auditory processing since rats continue responding to the CS in context A (Fig. 5D).

In order to rule out that the prolonged inactivation of the pIC is affecting the recall of fear memory, we transiently inactivated the pIC prior to CS presentation. In a different group of rats, MUS were infused into the pIC 2 h prior to test 3 (Fig. 6A). In contrast to the effect of prolonged inactivation, we found that the inactivation with MUS infusion had no effect on fear expression compared to SAL/pIC (Fig. 6, test 3, $t_{48} = 1.323$, $p > 0.05$), showing that the activity of the pIC is not necessary to recall conditioned fear. These data show that the inactivation of the pIC with NSTX, induced a protracted and progressive decrease in expression of conditioned fear, and suggest that the pIC is important for the long-term storage of fear memory.

3.4. Experiment 4. Expression or early genes accompanying fear memory reactivation.

Expression of conditioned fear to tone was paralleled by an increase in Fos-ir and Zif268-ir in the IC (Fig. 7), markers of neuronal activation and neural plasticity [13]. Fear-conditioned rats (conditioned group) showed a robust conditioned response to CS on day 2 compared to control rats (control group, Fig. 7A, $t_8 = 8.486$, $p < 0.0001$). The PL cortex, a cortical region known to be involved in the expression of conditioned fear Burgos-Robles and Vidal-Gonzalez [8] showed also an increased Fos-ir ($t_8 = 1.908$, $p = 0.0464$, Fig. 7B). In contrast, no significant activation was observed in the SSp ($t_8 = 0.045$, $p = 0.4826$, Fig. 7B), a cortical region that served as a control for unspecific cortical activation. Fos-ir was increased in the pIC (Fig. 7C), specifically at levels +0.95 ($t_{44} = 3.571$, $p < 0.05$) and +0.45 ($t_{44} = 4.837$, $p < 0.05$) from bregma. Conditioned rats also showed an increase in the expression of Zif268 (Fig. 7D), in the RAIC, at level +1.2 ($t_{44} = 3.657$), and in the pIC, at the levels +0.45 mm ($t_{44} = 4.986$), −0.26 mm ($t_{44} = 4.419$) and −1.78 mm ($t_{44} = 4.649$) from bregma. Taken together, these data show that reactivation of fear memory is accompanied by an increase in pIC neural activity and expression of early genes involved in neural plasticity, further supporting a role for the pIC in the long-term storage of fear memory.

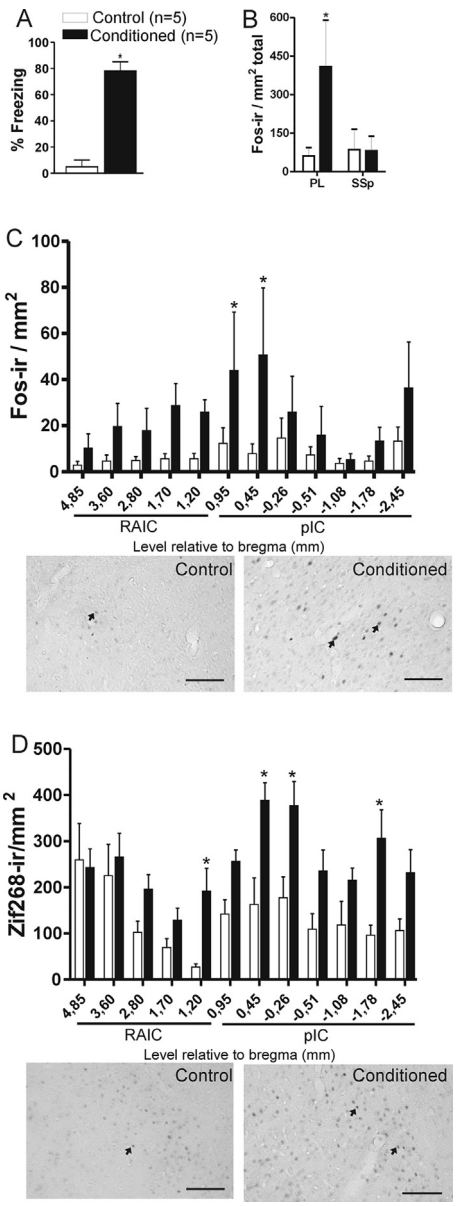

Fig. 7. Reactivation of learned fear was paralleled by an increase in the neural activity of the insular cortex (IC). A. Freezing to the tone in context B of the rats examined for early genes expression. Conditioned rats showed higher levels of freezing to the tone relative to unconditioned rats. B. Quantification of Fos immunoreactive cells in Prelimbic cortex (PL) and Primary somatosensory cortex (SSp) in control and conditioned rats. C. Upper panel: Quantification of Fos-ir in two different regions of the IC, the rostral agranular insular cortex (RAIC) and the granular field of the pIC, at different levels from bregma. Lower panels: Representative photomicrographs of the pIC showing the near absence of Fos immunoreactivity in control rats (left) and high Fos-immunoreactivity (arrow) in conditioned rats (right). D. Upper panel: Quantification of Zif268-ir in the IC at different levels from bregma. Lower panels: Representative photomicrographs of the pIC showing low Zif 268 immunoreactivity (arrow) in control rats (left) and high Zif268-immunoreactivity (arrow) in conditioned rats (right). Data are expressed as mean + SEM, $^*p < 0.05$, Two-Way ANOVA and Bonferroni post test. Scale bars indicate 100 μm.

4. Discussion

4.1. The role of the pIC in conditioned fear had not been studied

Although there are reports involving the IC in learned fear, they are either not specifically focused on the pIC [2,30] or deal with lesions of the IC alone or in combination with other structures [7,38], which hinder the interpretation of the results. In the present report we used reversible inactivation and protein synthesis inhibition in the pIC in order to contribute to clarify this issue. We found that pIC inactivation prior to training had no effect over fear expression during conditioning. However, a marked reduction in fear expression was seen 24 h after, during the extinction trials. Also, here we show that protein synthesis inhibition in the pIC immediately following training, but no 6 h after, also decreased fear expression during the extinction trials. These data indicate that the pIC is involved in the consolidation of fear memory. The participation of pIC in the long-term storage of learned fear is also supported by the long-lasting reduction in expression of conditioned fear observed following its inactivation with NSTX. Moreover, an increase in expression of early genes involved in neural plasticity that accompanies fear memory reactivation further supports this idea.

4.2. Methodological considerations

Saxitoxin and related paralytic shellfish toxins have been successfully used as a prolonged duration local anesthesia with minimal *in situ* toxicity [21] to treat a variety of ailments [35]. The main mechanism of action of these toxins is to block voltage-gated sodium channels [28,33]. The unusually long-lasting effect may be explained by the very low IC_{50} of 3.5 nM that this toxin has for blocking sodium voltage-gated channels [34] compared to other blockers such as lidocaine with an IC_{50} of 16 μM Castle et al., [11]. The low IC_{50} also should contribute to a limited diffusion of the NSTX. Our preliminary chronic recordings in rats from a sensory cortex infused with NSTX at the same concentration and volume used here, suggests that this toxin completely blocks action potentials within a radius of *ca.* 1 mm and for nearly a week, a time course similar to that in peripheral tissue [21]. A shortcoming of using NSTX to inactivate a brain region is that it blocks not only the local neurons but also the activity of axons passing through the injection site. While the radial disposition of axons coming in and out of the cerebral cortex [9] makes this issue less relevant, it is likely that in many other brain regions the passing axons may be a problem that will make the interpretation of focal inactivation with NSTX difficult. However, while NSTX into the pIC impaired the recall of the conditioned fear, the local infusion of muscimol had no effect on recall. This may be related to inactivation of fibers of passage, for instance from more rostral IC, in the vicinity of the NSTX injection site, or to some yet undetermined consequence of protracted inactivation of pIC.

4.3. Fear and the insula

The IC has been implicated in a number of cognitive functions [23]. Functional neuroimaging studies in humans consistently implicate the anterior subdivision of IC (high-order) in perception of emotional states and the posterior subdivision of IC in the processing of afferent information about bodily states [18]. Both subdivisions are strongly interconnected; therefore, a bidirectional flow of bodily-related information between the pIC to the RAIC seems plausible [12,39] and necessary for the emotional experience [19]. Moreover, the RAIC has strong bidirectional connections with the amygdala [39]. In line with this, Alves et al. [2] found that the reversible inhibition of the neurotransmission within the RAIC immediately after training or 10 min before re-exposure to the aversive context, attenuated fear expression. On the other hand, studies oriented to unveil the pathway carrying unconditioned stimulus-related information to the amygdala have shown that lesions of the IC do not affect acquisition of conditioned fear [7,24,38], but blocks fear-potentiated startle, [36], or prevent the mitigation by safety signals of the anxiety resulting from inescapable tail shocks [14,15]. Differences in methodology and restoration of function following lesions, may explain conflicting results. Nevertheless, these studies are focused on the most caudal IC, from 2 mm behind bregma, which is outside the limits of axonal projections from the parvicellular part of the ventroposterior lateral thalamic nucleus, the main thalamic relay of visceral information to the pIC i.e., between 0.0 to 1.5 mm behind bregma; [17,39]. The results from the present work are focused in the pIC region, defined above as the main target of the interoceptive thalamus, and this region had not been studied before in relation to learned fear.

We recently reported that transient inactivation of the pIC increases the latency to display escape behavior in rats submitted to severe hypoxia [10]. Similarly, in a previous work from our group we showed that transient inactivation of the pIC increases the latency to express gastrointestinal malaise and disrupts drug craving [17]. We have argued that a disruption in the processing of interoceptive signals takes place when the pIC is inactivated, affecting the distribution of information within the IC and the proper behavioral output. In the present work fear expression during conditioning and recall remained intact after pIC inactivation (Fig. 2, Fig. 6). However, a marked reduction in freezing was observed when the CS was presented 24 h later (Fig. 2). These data suggest that the IC is not involved in the execution of conditioned freezing, but that plasticity related to fear learning takes place in this cortex.

4.4. Insula and memory

Previous work clearly establishes that the IC is involved in the storage of long-term memory [5]. Besides its well established role in taste memory Bermudez-Rattoni [4], it has also been involved in drug-related memory [16] and recently, in contextual fear memory [2]. In the present report, we observed that the activity of the pIC following conditioning is important for the maintenance of fear memory, suggesting that consolidation of conditioned fear takes place in the pIC. Accordingly, anisomycin infusion into the pIC, immediately following training, significantly reduced fear expression 24 h later, indicating that plastic changes occur in the pIC during conditioning and that its stabilization after training is necessary for long-term storage of a fear memory trace into the pIC. Our results extend previous work regarding the role of the IC in fear memory, establishing a role of the pIC in consolidation of cue conditioned fear memory.

In support of this idea we found that the inactivation of the pIC with NSTX, immediately after reactivation of fear memory, progressively reduced fear to subsequent CS exposures. A possible explanation for this effect is that NSTX is persistently blocking the recall of conditioned fear, due to its long-term action. To rule out this possibility, we infused MUS bilaterally into the pIC 2 h before recall in test 3. This inactivation of the pIC had no effect in fear expression on test 3 or in any subsequent test, making an immediate effect in recall an unlikely possibility.

The effect of NSTX in expression of conditioned fear is similar to that observed when interventions are made within the reconsolidation window [32,25]. Ample evidence in reconsolidation establishes that following reactivation, memories are susceptible to modification causing long-term amnesia for fear memories [31]. Accordingly, we observed an increase in the expression of early genes involved in neural plasticity accompanying the reactivation of conditioned fear. These data suggest that inactivation of the pIC following reactivation impaired the reconsolidation of conditioned fear memory. More experiments are needed to properly address this issue.

4.5. Somatic changes during fear

Several physiological changes take place during fear conditioning and expression of fear, including effects on muscular activity [40], body temperature Vianna and Carrive [42], and the cardiovascular system [27]. This cluster of fear-related bodily states might be represented and stored in neural ensembles in the IC during fear conditioning, thereby increasing the perceptual sensitivity to the interoceptive changes that accompany this emotion. In this sense, it has been described that the central representation of an emotionally competent stimulus, such as a conditioned tone in the auditory cortex increases after learning, making it more easily perceived and distinguishable from similar stimuli [43]. Interestingly, the convergence of the information about tone and foot shock in the auditory cortex is necessary for fear learning [26]. Functional imaging studies indicate that IC is involved in several aspects of auditory processing [3]. The pIC receives strong auditory inputs from the ventral auditory cortex, an area implicated in auditory information processing related to emotion, and auditory evoked responses can be recorded from the pIC [22]. We think that the interoceptive rep-

resentation of a fear-related bodily state might become associated to the tone information within the pIC during fear learning.

4.6. In summary

The present results show that, following training, the activity and protein synthesis in the pIC are important for consolidation of fear memory. Also, we showed that long-term inactivation of the pIC induced a persistent reduction in subsequent reactivations of conditioned fear. These data add further support for a role of the IC in the regulation of fear memories.

Acknowledgements

We thank Paulina Arias for technical support. This work was financially supported by Milenio P10-063-F, Anillo Conicyt ACT-66 and Fondecyt 1130042.

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