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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.

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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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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.

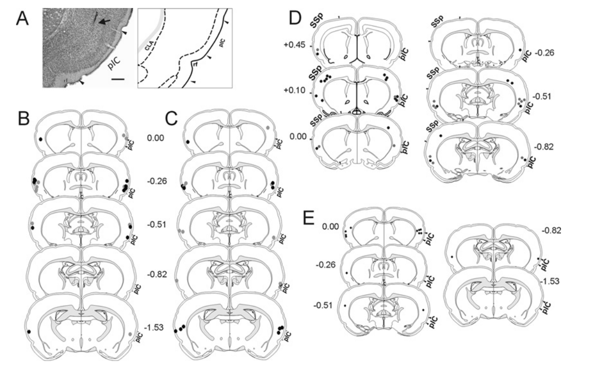

Fig. 1. Representative example of cannula placement in the posterior insular cortex (pIC). A. Left panel, photomicrograph of a Nissl stained coronal section showing the endpoints of the injection cannula tips (arrow) in the pIC, and a schematic drawing (right) modified from Swanson's atlas. B. Muscimol (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,