PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
Doctorado en Neurociencias

Tesis Doctoral

STUDY OF THE NEURONAL ACTIVITY IN THE INTEROCEPTIVE INSULAR CORTEX IN DEFENSIVE BEHAVIORS ELICITED BY INNATE FEAR INDUCER

por

MARIA DE LOS ANGELES RODRIGUEZ SOTO

Abril, 2018

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Tesis presentada a la Pontificia Universidad Católica de Chile como parte de los requisitos para optar al grado de Doctor en Neurociencias

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DEDICATORIA

A mi familia por su continuo amor y generosidad

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TABLE OF CONTENTS

LIST OF FIGURES.

LIST OF TABLES.

LIST OF ABBREVIATIONS.

ANS	Autonomic nervous system
AP	Action potential
BP	Blood pressure
CP	Caudate putamen
dl	Dorso lateral
dm	Dorso medial
EIB	Electronic integrated board
Fos-ir	Fos immunoreactivity
FR	Firing rate
GI	Gastro intestinal
HFR	High firing rate
HR	Heart rate
IC	Insular cortex
ir	Immunoreactivity
ISI	Inter spike interval
l	Lateral
INeu	Interneuron
LA	Lateral Amygdala
LFR	Low firing rate
MeA	Medial Amygdala
Mus	Muscimol
NSTX	Neosaxitoxin
NTS	Nucleus tractus solitarius
PAG	Periaqueductal grey matter
pIC	Posterior insular cortex
PL	Prelimbic cortex
pv	Posteroventral
RAIC	Rostral agranular insular cortex
rf	Rhinal fissure
Sal	Saline
SEM	Standard error of the mean
SS	Somatosensory cortex
vl	Ventrolateral
VMH	Ventromedial Hypothalamus
VPLpc	Ventroposterolateral parvocellular

RESUMEN

Varios estudios en humanos sugieren que el miedo y la ansiedad pueden estar relacionados con la función de la corteza insular (CI). Pocos estudios experimentales en ratas han involucrado la CI en respuestas de miedo. Recientemente, informamos que la inactivación, previa al condicionamiento a miedo, de la corteza interoceptiva primaria (CIp) o el bloqueo de la síntesis de proteínas de la CIp, inmediatamente después del entrenamiento, alteraba la consolidación del condicionamiento del miedo auditivo. Es por esto que esta tesis se formuló para investigar el papel de la CIp en respuestas defensivas tanto innatas como aprendidas. Se llevaron a cabo tres estudios para abordar este tema. Utilizamos un enfoque etológico que nos entrega una descripción detallada de comportamientos defensivos que reflejan miedo y ansiedad, los cuales son conductas de congelación y evaluación de riesgo respectivamente. Estas conductas fueron obtenidas en respuesta a un estímulo que evoca miedo y ansiedad de manera innata, el cual fue presentado en un collar impregnado con olor de un gato doméstico.

En el primer estudio, investigamos el papel del CIp en los comportamientos defensivos gatillados por tres condiciones: exposición única (innato), re-exposición al olor a gato y exposición al contexto (aprendido). Nuestros experimentos con Muscimol,

mostraron que la inactivación de la CIp, pero no de la corteza somatosensorial (SS), redujo la expresión de la conducta de congelación en respuesta a las tres condiciones antes mencionadas, siendo sólo alterada la conducta de evaluación de riesgo en condiciones de aprendizaje. Encontramos también que la inactivación a largo plazo de la CIp con Neosaxitoxin (NSTX), resultó en una reducción prolongada y robusta de la respuesta de congelación en exposiciones subsiguientes al olor de gato, además de observarse una reducción a transitoria en la conducta de evaluación de riesgos sólo 24h después de la inactivación. No observamos ningún cambio significativo en las conductas no defensivas. Estos datos sugieren un papel crítico de la CIp en la expresión del miedo innato, así como también la regulación de conductas defensivas y la formación de memorias de miedo y ansiedad.

El segundo y tercer estudio, fue desarrollado para determinar el patrón de actividad de la CIp en ratas expuestas al estímulo amenazante. Cuantificamos la inmunorreactividad de neuronas positivas para Fos (marcador de actividad neuronal) después de la presentación de este estímulo y realizamos registros electrofisiológicos extracelulares de unidades neuronales de la CIp, en ratas despiertas, durante las conductas de miedo y ansiedad innatas. Descubrimos que el olor a gato aumentó la actividad neuronal en la CI, tanto en la CIp como en la corteza insular anterior (RAIC), así como en las estructuras subcorticales clave involucradas en el circuito de miedo innato. También encontramos una fuerte correlación positiva entre la actividad neuronal de una discreta área de la CIp y el comportamiento congelación tras la exposición al estímulo amenazante. En el tercer estudio, encontramos diferencias en

cómo la CIp codifica la información interoceptiva de los estados de miedo y ansiedad. Observamos que el 35% de las neuronas cambió su tasa de disparo (TD) asociada con la conducta innata de congelación inducida por el estímulo en estudio. Esta respuesta mostró un cambio transitorio en el patrón de disparo de estas neuronas. Por el contrario, un 38% de las neuronas registradas cambiaron significativamente su TD durante el comportamiento de evaluación de riesgos, mostrando un cambio sostenido durante el transcurso de este comportamiento. Entre estas poblaciones encontramos neuronas que modifican su TD específicamente para cada comportamiento en particular, mientras que otras responden durante ambas conductas. Estos dos estudios en conjunto muestran que la conducta de congelación, pero no la evaluación del riesgo, se correlaciona significativamente con la actividad neuronal de la CIp, lo que sugiere que un cambio agudo en la TD durante el inicio de este comportamiento parece ser funcionalmente relevante dentro del circuito del miedo innato.

En resumen, nuestros tres estudios entregan datos que muestran por primera vez que la CIp está involucrada funcionalmente en la expresión del miedo innato y así como en la regulación de las respuestas emocionales al miedo aprendido.

SUMMARY

Several studies in humans suggest that fear and anxiety may be related to the function of the insular cortex (IC). Few experimental studies in rats have implicated the IC in fear responses. We have recently reported that pretraining inactivation of the primary interoceptive cortex (pIC) or the intra-pIC blockade of protein synthesis immediately after training impaired the consolidation of auditory fear conditioning. The present study was designed to investigate the role of the pIC in innate and learned defensive responses to predator odor. Three studies were conducted to address this issue, using an ethological approach that involves a detailed description of defensive behaviors, such as freezing and risk assessment (i.e fear and anxiety-related behaviors, respectively) elicited in response to predatory stimulus presented in a collar impregnated with odor obtained from a domestic cat.

In the first study we investigated the role of the pIC in defensive behaviors elicited by three conditions: single exposure (innate fear), re-exposure to cat odor and context exposure (learned exposure). Our experiments showed that pIC inactivation with Muscimol, but not somatosensory cortex (SS), reduced the expression of freezing

in response to a single or repeated exposure to cat odor. We also found that the pIC inactivation with Muscimol impaired conditioning of fear to the context in which rats were exposed to cat odor. Furthermore, Neosaxitoxin (NSTX) inactivation of the pIC resulted in a prolonged and robust reduction in freezing response in subsequent re-exposures to cat odor. A short-term reduction was observed in risk assessment after pIC inactivation with NSTX. We did not observe any significant change in non-defensive behaviors. These data suggest a critical role of the pIC in the expression of innate fear and as well as in the modulation of defensive behaviors and formation of fearful memory.

The second and third studies were conducted to determine the pattern of activity of the pIC in rats exposed to innate fear inducer. We quantified the positive Fos immunoreactivity (Fos-ir) neurons after stimulus presentation and we performed extracellular single-unit electrophysiological recordings from the pIC in awake rats, during both innate fear and anxiety behaviors. We found that cat odor increased the neuronal activity in the IC, in both pIC and anterior insular cortex (RAIC), as well as, in key subcortical structures involved in innate fear system. We also found a strong positive correlation between neuronal activity of a discrete part of the pIC and innate freezing behavior after cat odor exposure. In the third study, we found differences in how pIC encodes the interoceptive information of fear and anxiety states. We observed that 35% of neurons changed their firing rate (FR) associated with the innate freezing expression induced by cat odor. This response showed a transient change in the firing pattern of these neurons. In contrast, a 38% of neurons recorded significantly changed

their FR during risk assessment behavior showing a sustained change during the time course of the behavior. Between these populations we found neurons that modify their FR specifically for each behavior in particular while others are responsive during freezing and risk assessment behaviors induced by cat odor. Taken together, these data show that the expression of freezing, but not risk assessment, is significantly correlated with the neuronal activity of the pIC, which suggests that an acute change in the FR during the onset of this behavior appears to be functionally relevant within the innate fear system.

In summary our three studies provide data showing for the first time that the pIC is functionally involved in the expression, the perception of innate fear and regulation of emotional responses to fear.

1. INTRODUCTION

"*The oldest and strongest emotion of mankind is fear*" (HP Lovecraft, 1945)

"*Emotions determine the quality of our lives. They occur in every relationship we care about […] and can save our lives*" (P. Ekman, 2007)

Emotions have been a big topic of discussion along the history of humankind. There are several theories of emotions trying to explain this phenomenon in different academic fields.

James and Lange in 1889 proposed that an emotional stimulus, induces physiological changes (heart rate, blood pressure or skin conductance) without affecting consciousness and then those bodily responses are interpreted by the brain to produce the emotional experience (feelings) being specific for each emotion. This perspective was deeply influenced by Darwin's theory (1872) in his book the expression of emotion in man and animals. In contrast, Cannon and Bard through their studies in animal physiology, concluded that autonomic feedback is not necessary for emotional feelings and instead they held that emotions have highly similar autonomic responses (Friedman, 2010). Other studies have indicated that facial and bodily postures patterns evoke an emotional response that could be interpreted as a particular type of emotion (Ekman et al. 1983; Flack et al. 1999). Facial feedback theories, based on Darwin's

theory, identified patterns that correspond to happiness, surprise, sadness, fear, anger, and disgust, but debate continues about on whether these results support the specific autonomic activity of the emotion (Barrett, 2006; Ekman, 1994; Levenson, 1992). On one hand, a study suggests that only negative and positive emotions can be differentiated based on autonomic responses, but not necessary discrete emotions (Cacioppo et al., 2000). On other hand, another study showed a significant autonomic specificity of fear vs. anger (Stemmler, 2004).

Damasio in 1994 with his theory of *somatic marker* proposed that bodily information is involved in decision-making process as well as emotion, and suggested that bodily signals are represented and regulated in an emotion neuronal circuit that is formed by regions of the limbic system structures including ventromedial prefrontal cortex (VMPFC), somatosensory cortices, insula, and basal ganglia (Damasio, 1998)

To date something is clear: emotions could be understood as a set of programs that guide cognitive, physiological, and behavioral processes in response a specific type of stimulus, to preserve homeostasis. The tight relationship between the nervous system with the environment, either external or internal, allows us to perceive our ongoing state. The sensory systems give us information about what is happening around or inside of the body. Current knowledge in neurobiology implicates the *Interoceptive sensory system*, as a sensor of the physiological condition of the body. This sensory system, involving viscerosensorial regions distributed in the brainstem, thalamus and cerebral cortex and associated with autonomic motor control, provides the conscious perception the body, and the subsequent behavioral decision-making

(Critchley, et al, 2004, Damasio et al., 2000), which is closely associated with motivated behaviors to homeostatically regulate the internal state.

The conscious perception of the activity of interoceptive receptors (e. g. mechanoreceptors, chemoreceptors and baroreceptors), located in every structure of the body arises, we believe, from the interaction between the thalamus and cerebral cortex, a process termed Interoception. The insular cortex (IC), a cortical component of the interoceptive system, has been implicated in the processing of bodily signals (Cechetto and Saper, 1987; Saper, 1982, Yasui, et.al 1991) and in a number of cognitive functions besides interoception, including emotions, attention, empathy, memory, etc. (Damasio, et al, 2000; Kurth et al. 2010; Menon and Uddin, 2010).

Until now, the neurophysiological mechanisms of sensory interoceptive processing are poorly understood. A previous study from our lab shows that the inactivation of the posterior IC (pIC) disturbs both drug craving and the gastrointestinal malaise induced by LiCl (Contreras et al., 2007); in the lab it was also found electrophysiological correlates of the pIC in the perception of the stomach malaise induced by LiCl (Aguilar-Rivera, 2012); concluding that these interoceptive states critically depend on the function of the pIC (Contreras et al., 2007). These data suggest a role for the insula in the perception of bodily and emotional states. On this basis, we hypothesized that the neuronal activity of the pIC represents the perception of different bodily states in response to an emotional stimulus.

Fear and anxiety elicit defensive behavioral responses that ensure the organisms survival since these behaviors are effective in avoiding or reducing harm in dangerous situations (Blanchard et al., 2001; Gross and Canteras, 2012). Studies have

indicated that fear is the predominant response to a realistic threat whereas anxiety is the reaction to a potential threat (Blanchard et al., 1991). Those behavioral responses are called defensive behaviors and are in part the result of the activity of a neuronal circuit that responds to predator, which consist of stereotypical behaviors and cardiovascular responses. Defensive behaviors are seen in different animal species and they are rapidly associated to stimuli and situations related with threat. Both determined by the defensive distance and the possibility to escape the situation (Blanchard et al., 1991) and include posturing in the crouched position, retraction of the head and ears, vocalizations, and an incremented sympathetic response (piloerection, pupillary dilation, heightened alertness, increased blood pressure, heart rate, dilation of vessels in skeletal muscle and widespread veno-constriction, Jordan, 1990; Dielenberg, 2001). This preparatory reflex sets the stage for maximal action where the pIC, an important site of interoceptive representation, should have a role for awareness of bodily state associated innate defensive behaviors. This leads us to formulate the following hypothesis: *"The activity of the interoceptive insula of the rat is necessary for the expression and perception of innate defensive behaviors"*. To test this hypothesis, we evaluated the role of the insula in the expression and perception of innate defensive behaviors induced by predator odor. We evaluated how fear but also anxiety expressions are represented as neuronal activity in the insula. The results from these studies are discussed mainly in terms of the interoceptive function of the insula.

1.1. Interoceptive system

The brain is continuously receiving information coming from a large variety of receptors that sense physiological processes and states of the body. This function is carried out by the interoceptive system, which sends bodily information (e.g. arterial blood pressure, arterial oxygen pressure level, gastric distension, etc.) throughout their afferents to the brain. This information is processed by the ascending interoceptive pathway (Acuña-Goycolea et al, 2000), which is formed by interconnected brainstem and thalamic regions before reaching the IC. It has been proposed that the physiological changes promote behavioral decisions in order to maintain the internal homeostasis (Saper, 2002). There are three possible routes by which interoceptive information can feedback to the brain and influence in behavioral decisions. One is by cranial nerves (VII, IX and X) to the nucleus tractus solitarius (NTS) located in the medulla, another is by afferents in spinal nerves that innervate skin and skeletal musculature, and project to Lamina I of the spinal cord and a third is an endocrine route by a complex formed between the NTS and the Area Postrema (AP).

The visceral and cardiovascular information, through cranial nerves, enter to the brain by NTS in a topographically organized manner and then this information reaches the parabrachial nucleus (PBN) of ipsilateral way in the brainstem (Saper, 2002). PBN neurons project axons to the ventroposterolateral parvocellular (VPLpc) of the thalamus interoceptive opposite, a structure that sends projections to the IC, mostly to the granular region of the primary interoceptive posterior insular cortex (pIC, Cechetto and Saper, 1987, FIGURE Nº1). The pIC receives sensory information from a large variety

of interoceptive receptors distributed in nearly every structure of the body and distributes information to anterior regions of the insula and to other IC regions that are connected to the executive prefrontal cortices, including the medial prefrontal cortex, the orbitofrontal and anterior cingulate (Shi and Cassell, 1998). A comparable posterior-anterior IC relationship has been described in primates (Mesulam and Mufson; 1985) and humans (Critchley and Harrison, 2013). The above show us that this system provides behavioral decisions to regulate homeostasis.

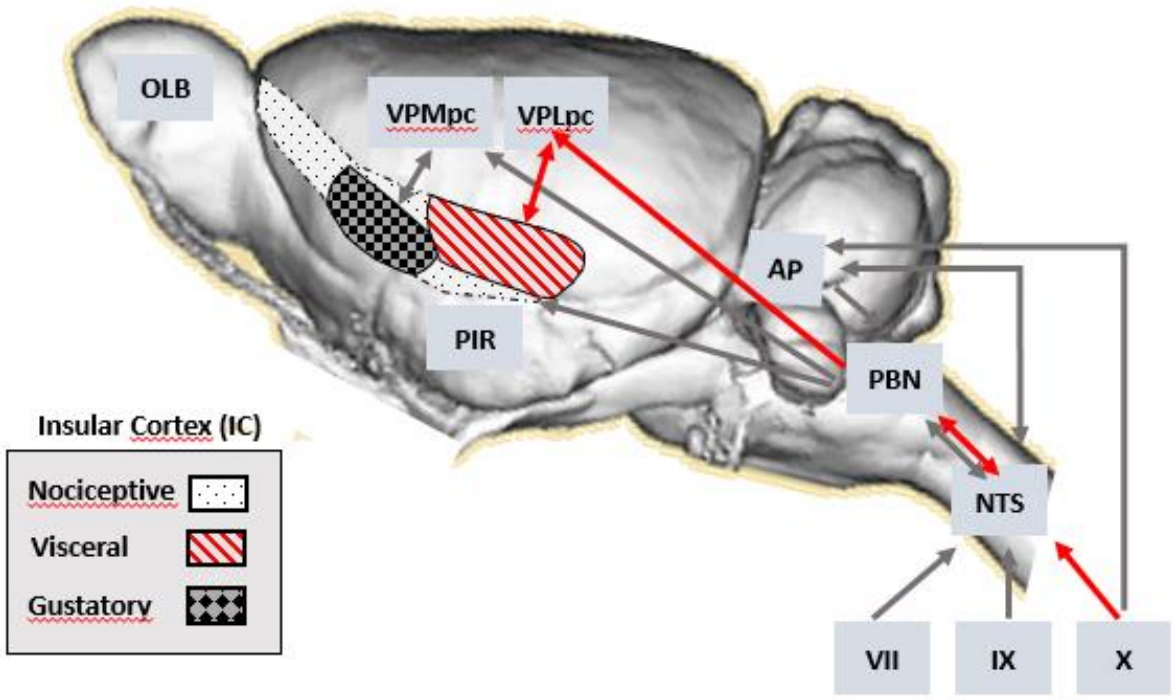

FIGURE Nº1. Interoceptive flow of neuronal signals from the body. A lateral view of the rat insula showing connections of the IC with two possible routes of ascending interoceptive information. Red arrows showing visceral sensory system, including cranial nerve X (vagus nerve), NTS (nucleus tractus solitarius), PBN (parabraquial nucleus), and VPLpc (ventroposterolateral parvocellular) of the thalamus before to reach pIC (Ascending pathway studied here). Cranial nerves VII and IX activating gustatory and nociceptive sensory systems by VPMpc (ventroposteromedial, parvocellular) of the thalamus or PBN (parabraquial nucleus). The other one is an endocrine route by a complex formed between the NTS and the Area Postrema (AP). Surrounding insula brain structures: OLB (olfactory bulb), PIR (piriform cortex). The ascending pathway for the Lamina I is not shown. Adapted from Oppenheimer and Cechetto, 2016.

1.2 Insular Cortex

The IC is a region of the cerebral cortex found in the depth of the lateral sulcus - a fissure separating the temporal lobe from the parietal and frontal lobes - in humans and monkeys. In rodents, this cortex surrounds the rhinal sulcus. The IC was first described by Johann Christian Reil (Reil, 1809), after which it received the name “the island of Reil”.

The earliest studies documenting a sensory representation in the IC was conducted by vagus nerve stimulation of cats (Bailey and Bremer, 1938; Dell and Olson, 1951). These authors studied the different anatomical connections of the NTS and its effects on cortical activity and demonstrated a region of vagal receptive cortex that corresponding to the IC. Penfield in the 1950s, by electrical stimulation in patients’ ongoing neurosurgical procedures, identified regions into the IC that produces the sensation of taste, feelings in the oropharyngeal, esophageal or gastrointestinal tracts (Penfield and Faulk, 1955; Penfield and Rasmussen, 1950). It should be noted, these subjects did not felt complete emotional responses by stimulation into the IC, but even so, they made a variety of descriptions about their visceral sensory experiences.

Electrophysiological evidence in anesthetized rats has shown that the pIC is an important site of autonomic representation. Within the rat insula a viscerotopic organization within the rostro-caudal dimension of the pIC has been identified, with a rostral insular site receiving mechanoreceptor, gustatory and gastric inputs; and a caudal insular region receiving baroreceptor and cardiac inputs (Cechetto and Saper,

1987). Studies have shown changes in the firing rate of the neurons into the posterior insular cortex in response to the baroreceptor stimulation (i.v. phenylephrine or nitroprusside) and chemoreceptor (i.v. cyanide or 10% CO2). Further, the electrical stimulation of the pIC induces changes in the mechanoreceptors of lung, gastrointestinals and viscera, as well as, in the cardiovascular system either increasing or decreasing blood pressure (BP) or heart rate (HR, (Cechetto and Saper, 1987; Hanamori, 2005; Yasui et al., 1991, FIGURE Nº2). A recent study by Rizzolatti group (Caruana F., et al., 2011) identified a behavior that reflects disgust (a basic emotion) and pro-social behavior, both motor and autonomic component characteristic by microstimulation of the IC in the monkey. The above results located the IC as an important structure of the brain in the awareness of visceral sensations.

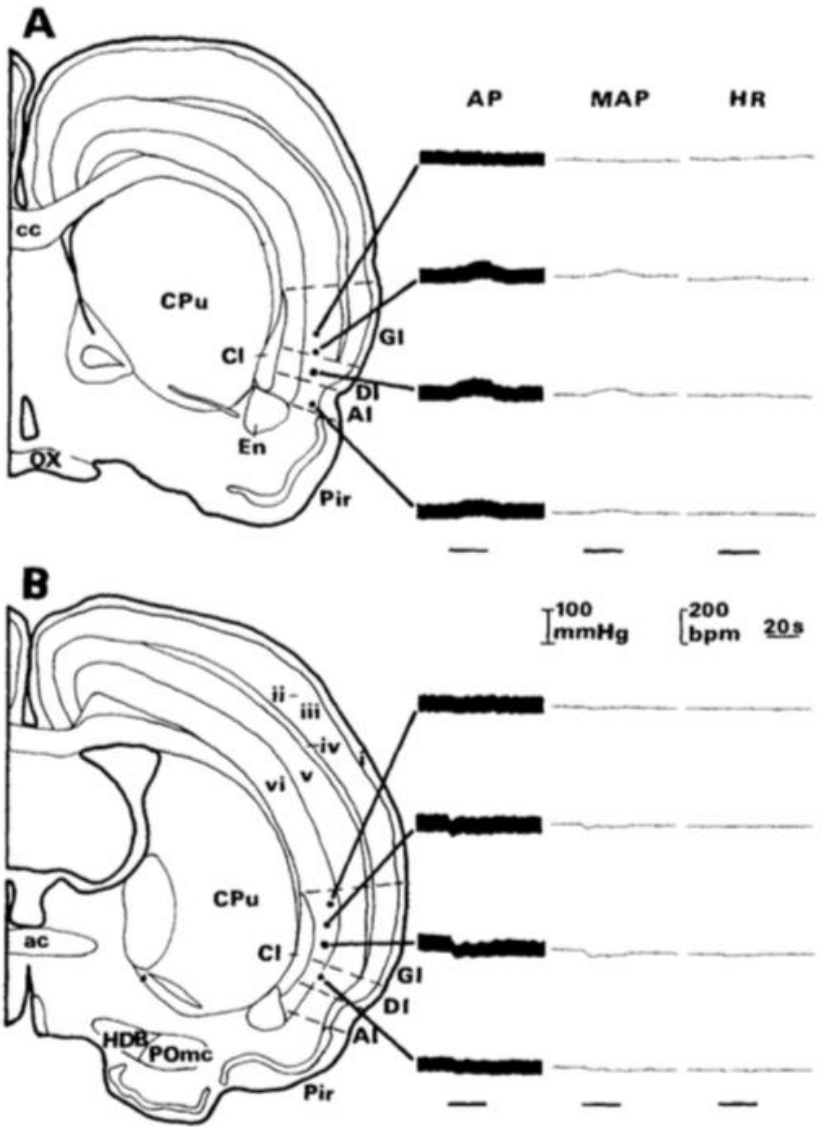

FIGURE Nº2. Electrical stimulation of the IC in the rat elicits a cardiovascular response. A. Illustrates a site from which an increase in arterial pressure is accompanied by tachycardia. B. Illustrates a depressor response and bradycardia obtained from layer VI of the ventral granular posterior insular cortex. In each case the first column shows the pulsatile arterial pressure (AP), the second the mean arterial pressure (MAP) and the third the heart rate (HR). Stimulation consisted of 1 ms pulses delivered at 50Hz for the time indicated by the bar under each column. i-iv, cortical layers. Adapted from Yasui et al., 1991.

Imaging studies in humans have revealed a participation of the IC as well as a number of subcortical structures in several phenomena related to emotions. As background we can mention the IC participation in the recall and re-experience of different emotions (Damasio et al., 2000), in interoceptive sensitivity (precision for heartbeat timing) and the susceptibility to experience emotions (Critchley et al., 2004). In addition, it has been reported that damage to the IC produces a selective deficit in the recognition of facial expression of disgust (Calder et al., 2000).

Other human studies have reported that lesions in the IC caused by cerebrovascular accidents have been associated with myocardial hypertrophy, arrhythmias and sudden death (Abboud et al., 2006; Laowattana et al., 2006; Oppenheimer et al 1991).

All these data suggest a role of the IC in conscious perception of the many interoceptive signals that occur during an emotional state ongoing, so it may be a neurobiological substrate to experience emotional feelings. We think that the processing of interoceptive information within the insular circuits is necessary for the modulation of physiological responses to stimulus affecting homeostasis. In the present work we evaluated the role of the insula, particularly of the pIC, in the regulation of behavioral responses to innate fear inducer. We tested the idea that the activity of the pIC is associated to the behavioral expression of fear, which give us an idea of the contribution of interoceptive signals processed by pIC during fear experience.

1.3 Defensive behaviors as emotional responses

Defensive behaviors are a set of responses trigger by recognizing of a threat, improving an animal's chance of survival in confrontations with threat: predators or conspecifics. These behaviors are evolutionarily conserved across numerous species as birds, rodents, monkeys, and humans, among others (Blanchard et al., 2001; Gross and Canteras, 2012).

It has been proposed that these behaviors can be classified depending on the immediacy and intensity of threat stimulus and the ability to regulate their responses is essential to adaptation and survival (FIGURE Nº3). First, when an imminent threat source is present flight or freezing is the primary response pattern. The selection of flight or freezing is based on two factors: predatory distance and the availability of an escape route. Freezing is most often observed when threat stimulus is located at a distance and/or if an escape route is not available and categorized as behavioral measure of fear (Blanchard and Blanchard, 1972). In other hand, in situations in which the threat source is potentially dangerous or novel the animals show a specific behavioral pattern known as risk assessment (Blanchard et al., 2011), which is responsive to anxiolytic agents and has been used as index of anxiety.

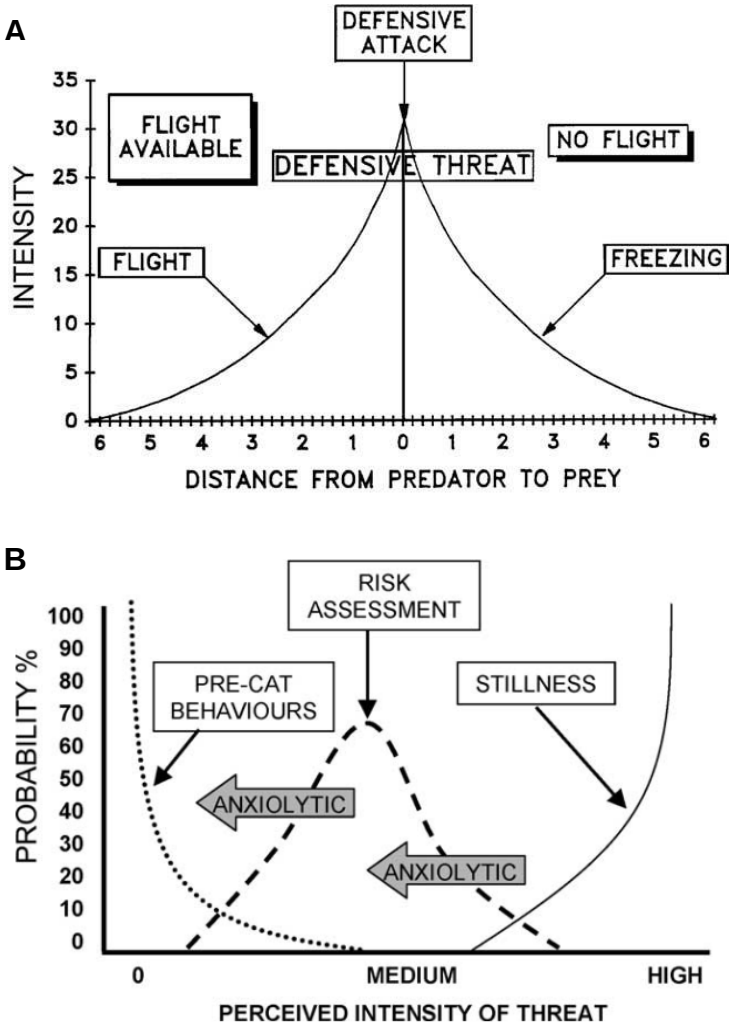

FIGURE Nº3. Relationship between defensive distance and behavior. A. As distance from a predator decreases, the intensity of fear increases. This intensity controls very tightly a progression from flight or freezing (depending on whether flight is available). B. For intermediate distances, risk assessment behavior occurs and, at very great distances, defensive behavior disappears and normal pre-threat behavior reappears The grey arrows represent a fixed change in defensive distance produced by anxiolytic drugs both increasing and decreasing risk assessment behavior depending on the initial defensive distance. Adapted from McNaughton and Corr, 2004.

Stimuli such as predators, aggressive members of the same species, pain, and dangerous features of the environment such as heights elicit fear responses. These stimuli markedly and consistently induce defensive behaviors that do not depend on the experience of direct injury associated with the threat or on a learning process setting an appraisal of danger to the threat. This type of fear is what has been referred to as “innate fear” (Blanchard and Blanchard 1989; Dielenberg et al., 2001). However, an innate fear experience also induces the formation of a memory of the fearful event. This process generates long-lasting changes in the brain in order to decrease the possibility to reencountering the same threat and to deal in a better way with similar future events. The association between the innate fear stimulus and a neutral stimulus such as, for example, the context where that stimulus was met is one component of this memory. This has been called "conditioned or learned fear”

Different anatomical pathways beyond learned and innate fear response have been suggested (Gross and Canteras, 2012). It is thought that subsets of sensory neurons are genetically determined to mediate innate behaviors. In rodents, olfactory and vomeronasal stimulation from a predator are critical to elicit an innate fear response (Papes et al., 2010) that consists of specific behavioral, endocrine and neuronal changes (Blanchard et al., 1998; Dielenberg et al., 2001, 2004; McGregor et al., 2004; Gross and Canteras, 2012).

Studies in rodents have involved a subnucleus of the amygdala, Medial Amygdala (MeA) as important region in the innate fear circuit. Rats exposed to a natural predator or its odor show substantial activation in this nucleus and rats with

lesions in the MeA show a reduction in innate fear responses to cat odor (Li et al., 2004). The olfactory and vomeronasal cues convey though their direct connections from principal and main accessory olfactory bulb to the MeA (Canteras et al., 1995).

The information coming from the MeA conveys to a subnucleus of the ventral hypothalamus (VMH), specifically in the dorsomedial area (dmVMH) where starts a predator-response circuit of the medial hypothalamic zone (FIGURE Nº4, Gross and Canteras, 2012).

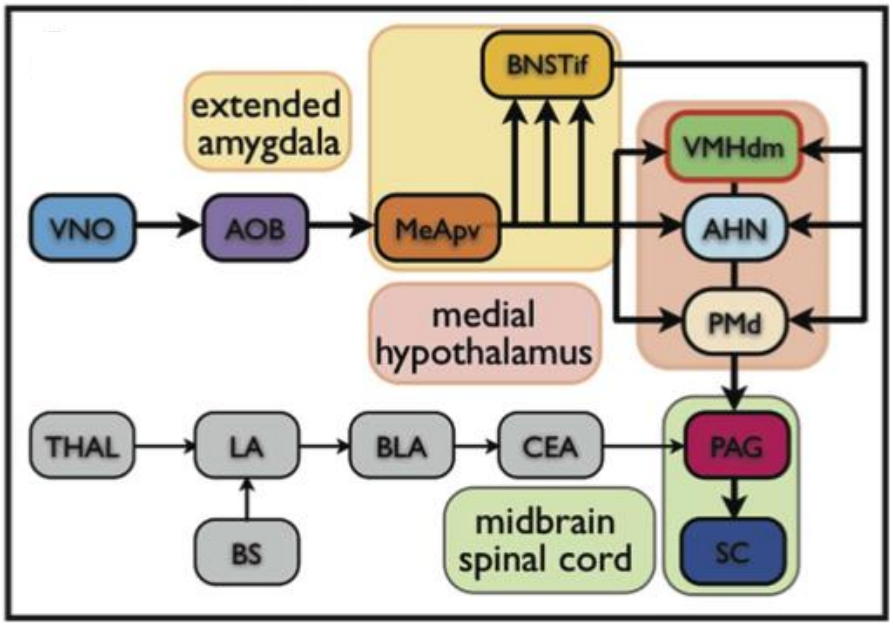

FIGURE Nº4. Schematic illustrating brain circuits involved in defensive behaviors in response to predator odor. A. indicates the innate fear defensive circuit. B. indicates learned fear defensive circuit. Brain structures: Vomeronasal organ (VNO), accessory olfactory bulb (AOB), posteroventral subnucleus of medial amygdala (MeApv), interfascicular nucleus of the bed nucleus of the stria terminalis (BNSTif), dorsomedial subnucleus of ventromedial hypothalamus (VMHdm), anterior hypothalamic nucleus (AHN), dorsal premamillary (PMd), periaqueductal gray (PAG), spinal cord (SC), thalamus (THAL), lateral amygdala (LA), basolateral amygdala (BLA), central amygdala (CEA). Adapted from Kunwar et al., 2015.

Studies have shown that during defensive behaviors occur changes in autonomic parameters, such as increased HR and BP (Le Doux, 1988; Dielenberg and McGregor, 2001), an increase plasma glucocorticoid levels (Figueiredo et al., 2003), effects on muscular activity (Steenland and Zhuo, 2009), body temperature (Vianna and Carrive, 2005), among others. Thus, is believed that medial hypothalamic zone is responsible of the regulating the autonomic response presented in the emotional response evoked by the predator odor (Gross and Canteras, 2012).

In brief, we think that these interoceptive and humoral signals associated to defensive behaviors, they would be sensed by the interoceptive system. These signals would be processed in the insular cortex that receives the information sent from the thalamus interoceptive. This information represented in the insular cortex would show the neuronal codes for fear and anxiety, which could be important in behavioral decisions taken by the animal to regulate its homeostasis.

1.4 Fear / Anxiety and pIC

Neuroimaging human studies have involved a role of the IC in both fear and anxiety (Damasio et al., 2000; Phelps et al., 2001, 2004; Morris and Dolan, 2004; Alvarez et al., 2008; Paulus and Stein, 2010).

In rodents, anatomical studies have been reported that the IC is connected to key structures for defensive behaviors, such as the medial amygdala, the medial prefrontal cortex, the ventromedial hypothalamus and downstream midbrain, either

directly or by other structures of the interoceptive pathway (Canteras et al., 1994; Canteras, et al., 1995; Shi and Cassell, 1998). However, the neurophysiological representations of sensory interoceptive processing and the possible relationship of interoception at the cortical level with emotional feelings have not been explored in detail.

Very few studies have been done on the role of the rodent's IC on fear. One study in rats has shown that the inactivation of the rostral IC region attenuates the consolidation of behavioral and cardiovascular responses to context after fear conditioning (Alves et al., 2013), but does not interfere with the acquisition of aversion to that context. The lesion of the most caudal IC after training blocked fear- potentiated startle (Shi and Davis, 1999). When comparing these studies, it seemed that one key difference lies on the specific IC region that was intervened. On the other hand, none of these studies is focused in the interoceptive region of the pIC, from +0.95 mm to -1.50 mm from bregma, where the densest projections from the interoceptive thalamus are found (Shi and Cassell, 1998; Contreras et al., 2007). Another recent work from our laboratory shows that the inactivation of pIC reduces the consolidation of auditory learned fear, but does not interfere with acquisition or fear expression (Casanova et al., 2016). However, the role of the IC in innate fear remains unknown. However, the role of the IC in defensive behaviors remains unknown.

A single study has revealed a discrete but significant increase of the neuronal activity in the IC in rats exposed to cat fur (Reed et al., 2013). While a study indicates that the functional activity of insular is essential for anxiety regulation (Li et al., 2014).

Another study done by Klarer and colleagues (2014) suggested a link between emotional responses such as fear and anxiety and the IC; showing that innate anxiety and learned fear are modulated by interoceptive flow as shown by subdiaphragmatic vagal deafferentation.

In the present work, we studied defensive responses by means of classical paradigm of predator odor exposure. It consists in the presentation of cat odor that triggers behavioral, bodily and neuronal changes. As we had defined, fear is an emotional response elicited by a stimulus that threats the integrity of an organism (Blanchard and Blanchard, 1972), while anxiety is a response in front a source potentially dangerous or novelty (Blanchard et al., 2011). The ability of animals to innately recognize the danger - as the presence of a predator - represents a basic survival mechanism. When the animal smelling its predator odor experiences a state that stimulates avoidance by the escape, freezing behavior, risk assessment behaviors or make disappear the stimulus that put him in a threatening situation, these behaviors are accompanied by bodily changes characteristic of this emotional response, such as cardiovascular changes, among others (Dielenberg et al., 2001). Fear and anxiety cause a variety of reactions depending on the intensity, timing, and coping options available, see TABLE Nº1, appendix table section. In this sense, the interoceptive system would be continuously updating homeostatic and emotional information, which is important for the expression of defensive behaviors.

2. HYPOTHESIS

Our main objective is to determine the role of the pIC in the innate fear. **Here we examined the hypothesis that the neuronal activity of the rat interoceptive insula is necessary for the expression of defensive behaviors to an emotional stimulus such as predator odor.**

Information about the physiological state of the body reaches the pIC, where the information is processed and distributed to other brain regions, in order to regulate autonomic variables and to display an appropriate behavior. The pattern of activity of the pIC during innate fear expression constitutes the neuronal correlate for the behavioral expression of this emotion. Accordingly, the pIC is responsible for the expression of fear genetically programed, which is crucial for the perception and behavioral expression of fear.

This hypothesis is based on the following observations: First, the pIC is the region of the IC with the densest projections from the visceral sector of the thalamus (Saper, 1982). Second, neurons from the pIC respond to several interoceptive stimuli (Cechetto and Saper 1987, Yasui et al, 1991, Hanamori, 2005). Third, the pIC is strongly interconnected with brain structures that regulate homeostasis and emotional responses (Saper, 1982). Fourth, the inactivation of the pIC delays the innate

behavioral expression of GI malaise induced by LiCl administration (Contreras et al., 2007). Fifth, the electrical activity of the pIC is associated with the behavioral expression of GI malaise. Sixth, the modulation of the innate anxiety and learned fear by the interoceptive flow that reaches the IC (Klarer, et al., 2014). Seventh, there is a discrete but significant increase of the neuronal activity in the IC in rats exposed to cat fur (Reed et al., 2013).

3. OBJECTIVES

General Objective

To study the relevance of the Interoceptive Insular Cortex (pIC) in the representation of innate fear in rats.

Specific Objectives

a. Determine whether the inactivation of the pIC decreases innate defensive behaviors by pharmacological manipulations.

a.1 Evaluate the effect of short-term pIC inactivation on innate defensive behaviors using Muscimol, a GABAA-R agonist.

a.2 Evaluate the effect of long-term pIC inactivation on defensive behaviors using Neosaxitoxin (NSTX), a lasting (a week) voltage-gated sodium channel blocker, on repeated exposure to cat odor.

a.3 Evaluate the effect of pIC inactivation on context to cat odor using Muscimol, a GABAA-R agonist

b. Determine the pattern of activity of the pIC and other relevant brain regions during cat odor using the expression of c-fos.

b.1 Determine the Fos immunoreactivity of the pIC during cat odor exposures.

b.2 Evaluate whether the retest to cat odor modifies the Fos immunoreactivity of the IC.

b.3 Determine whether the Fos immunoreactivity of the pIC correlates with defensive behaviors.

c. Determine the pattern of activity of the pIC using electrophysiological recordings.

c.1 Evaluate the single-unit activity in the pIC underlying innate defensive behaviors

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4. MATERIALS

Subjects. We used male adult Sprague-Dawley rats from the Faculty of Biological Science, weighing 270 g. at the beginning of the procedures. Rats for the behaviorally innate fear experiments were individually housed and kept in an inverted 12/12 h light/dark regime (lights on at 7:00 P.M). All test occurred during the dark cycle. Prior to the experiments the rats are handled for 5 consecutive days per 20 min for habituate to the experimenter.

They were kept in individual cages with access restricted free access to food and water, under controlled temperature (23-25ºC) and with a cycle of light / dark cycles of 12 h cycle (lights on from 8:00 A.M. to 8:00 P.M.)

Drugs. Anesthesia: Ketamine (44 mg/Kg i.p.; Imalgene, Aventis Pasteur SA, Santiago, Chile), Xylazine (3 mg/Kg i.p.; Rompún, Bayer, Santiago, Chile). Atropine (1 mg / kg) for reducing secretions. Antibiotic: Enrofloxacin 5%; 19 mg/kg i.p., Bayer). Anti-inflammatory: Ketoprofen 1% (Ketophen 0.2 mg/kg i.p., Rhodia Merieux). Muscimol (0,5 µg/ µl / side; Sigma-Aldrich, USA), Neosaxitoxin (32.5 µM/1 µL/side; CRM-MRCBiotoxin, Canada).

Cortical infusion of drugs. Stainless steel guide cannulae with removable occluders (4.5 mm long, 26 G; Plastics One, USA) were used to directly infuse drugs into the pIC. Injection cannulae (33 G; Plastics One) coupled to a 10 µL Hamilton syringe by a polyethylene tubing (inner diameter 1.27 mm; Plastics One) were used for infusions

Recording of neuronal activity. Tetrodes were made with NiCr wires (17-µm diameter) that were acquired from California Fine Wire Co. (USA). Commercial hyperdrives (Harlan 8, Neuralynx, USA) that allows individual manipulation of 6 tetrodes were used. A commercial headtage (HS-36, Neuralynx) was used to preamplify signals from the rat brain. A 32 channel computer-controlled setup with four Lynx-8 amplifiers (Neuralynx) was used to acquire neuronal signals. Custom software written in MATLAB was used for spike sorting and data analysis.

5. METHODS

5.1 General

Surgical procedures for implantation of infusion cannulas into the pIC. Rats were anesthetized with 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. Guide cannulae (26 gauge, Plastics One, VA) were fixed to the skull with screws (Plastics One, VA) and dental acrylic; an occluder sealed each guide cannula. The guide cannulae were targeted at the following coordinates according to Swanson's atlas (Swanson LW, 1998): Primary interceptive cortex (VISC, in Swanson's nomenclature), bregma -0.51 mm, midline 5.0 mm, depth from the cranial surface 4.5 mm; angled 10° from the vertical; somatosensory cortex (SS, in Swanson's nomenclature), bregma −0.46 mm, midline 4.5 mm, and depth from the cranial surface 1.5 mm. The injection cannula (33 gauge, from Plastics One, VA) protruded 2 mm beyond the tip of the guide cannula. The placement of the injection was verified by analyzing the location of the tip of the cannulae in Nissl stained sections. Antibiotics were administered at the end of the surgery (Enrofloxacin 5%; 19 mg/kg i.p., Bayer) together with a single dose of the anti-inflammatory ketoprofen 1% (Ketophen

0.2 mg/kg i.p., Rhodia Merieux). Rats were allowed to recover for 1 week before beginning behavioral testing.

Cortical injections. The injection cannula was coupled to a 1 µl Hamilton Syringe, filled with Muscimol (0.5 µg/ µl, Sigma-Aldrich) or sterile saline and inserted into the guide cannula after removing the occluder. We injected 0.5 µL/side during two minutes, slowly removed the injection needle, and replaced the occluders back immediately after the microinjection. For Neosaxitoxin dihydrochloride (NSTX)-treated rats, the injection cannula was coupled to a 10 µl Hamilton Syringe by a polyethylene tubing filled with this voltage-gated sodium channels blocker diluted in sterile saline (NSTX; 32.5 µM/µl/side, CRM-MRCBiotoxin, Canada) or filled with sterile saline. We injected 1 µl of NSTX or sterile saline during two minutes on each side slowly removed the injection needle; and replaced the occluders back immediately after. It has been shown that the action of Muscimol is up to 9h (Krupa et al., 1999) whereas NSTX completely abolished neuronal activity for approximately one week (Casanova et al., 2016; Epstein-Barash et al., 2009). Our preliminary electrophysiological data showed that the neuronal activity was strongly suppressed up to 1 mm after 1 µl of NSTX (32.5 µM) injection in the SS.

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-X100, 0.02

% sodium azide, 3 % normal goat serum in PBS) solution for 1 hour. The sections were then transferred to the primary antibody incubation solution, and left there overnight at room temperature. This incubation solution contained the Fos antibody (rabbit polyclonal F7799, from Sigma, Saint Louis, MO) diluted 1:20.000 in the blocking solution. The sections were rinsed in PBS for 1 hour before being incubated in the secondary antibody solution (Biotin-SP- conjugated AffiniPure goat anti-rabbit IgG (H+L) from Jackson ImmunoResearch, PA; diluted 1: 1,000 in 0.4 % Triton X100, 1.5 % normal goat serum in PBS). After rinsing for 40 minutes, the sections were incubated for 1 hour in Vectastain ABC Elite kit (Vector Laboratories, CA) diluted 1:500 in PBS, rinsed and incubated in a 0.05 % 3-3' diaminobenzidine hydrochloride (DAB) solution containing 0.003 % H_2O_2, and 0.05 % nickel chloride to get a dark blue reaction product.

Cell counting. The number of Fos-ir neurons was determined in coronal sections with the help of a camera lucida and an x10 objective. The size of the counting grid was related to the size of the selected area. For the rostral agranular IC (RAIC), from bregma +4.85 to +3.60, we used a 0.25 x 1 mm counting grid, from bregma +2.80 we used a 0.5 x 1.25 mm counting grid and from bregma +1.70 to +1.20 we used a 0.5 x 1 mm counting grid. For the pIC, from bregma 0.95 to -0.26 we used a 0.25 by 1 mm counting grid, and from bregma -0.51 to -2.45 mm, we used a 0.5 x 1 mm counting grid. For the posteroventral subnucleus of the medial amygdala (MeApv), from bregma -2.45, the dorsomedial subnucleus of the ventromedial hypothalamic nucleus (VMHdm),

from bregma -2.00 to -2.85 and the lateral amygdala (LA), from bregma -2.45 to -3.90, we used a 2.42 x 1.94 mm counting grid.

Design, assembly and tetrode loading of microdrive. Extracellular recordings were performed with custom-made tetrodes mounted in a commercial hyperdive. This arrangement of electrodes provides a more reliable way to identify signals from individual neurons than single wire electrodes (Gray et al. 1995; Harris et al. 2000). Briefly, two segments (14 cm) of polyimide insulated NiCr (80/20%) wire (17 µm diameter) folded on themselves were twisted. Heat (250 ^{o}C) was applied with a heat gun to facilitate adhesion between wires to keep this configuration. The tetrodes were inserted in polyimide tubes (Small Parts, USA) to protect them. A bundle of six stainless steel tubes (HTX-30T-30, outer diameter 300 µm; Small Parts, USA) of 2.5 cm each, was assembled within a Harlan 8 hyperdrive. Six tetrodes are inserted in the bundle (one for each tube) and were fixed to one of the 6 drives from the hyperdrive. Subsequently the electrodes will be exposed to 75% ethanol to prevent any further infection in the animal and then leave saved and placed aligned to come out of its sheath. Each wire from the tetrode was connected to an electrode interface board (EIB 36, Omnetics, USA), which provides the signal connection between tetrode wires and a 36-channel headstage pre-amplifier. A single silver wire (200 µm diameter; A-M systems) removed from its Teflon cover was attached to the EIB 36 board in one end, and tied to a small screw (TX1-3-C; Small Parts, USA) to be anchored in the skull as animal ground. Once it was checked there were no short circuit, the tip of each tetrode

was gold-plated by passing a cathodal current of 10 μA to low the impedance around 1-1.5 MΩ. For recordings, a headstage pre-amplifier containing 36 channels of unity-gain amplification (HS-36, Neuralynx) was connected to the EIB. The headstage was connected to a 32-channel, computer-controlled system (Four units of the Lynx-8 amplifier, Neuralynx).

Surgical procedures for implantation of tetrodes into the pIC. 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 with a microdrive (Neuralynx) aimed to the pIC using the following coordinates: Bregma, -0.51 mm, midline, + 6.0 mm, and -6.5 mm from the cranial surface, according to the Swanson's atlas (Swanson,1998). The microdrive consists of 6 independently movable tetrodes. Tetrodes were constructed with four nickel-chrome (17 μm) wires twisted together with an impedance of 1-1.5 MΩ (adjusted by gold plating procedures). The microdrive was fixed to the skull with 3 screws stainless- steel screws (Plastics One) and dental acrylic (FIGURE Nº5). One of those screws was used as animal ground. Right after surgery and for the following 3 days, rats were injected with 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. Tetrodes were lowered 480 μm daily (3 turns per screw), in 40 μm steps, until they reach the pIC at *ca.* 4000 μm under dura.

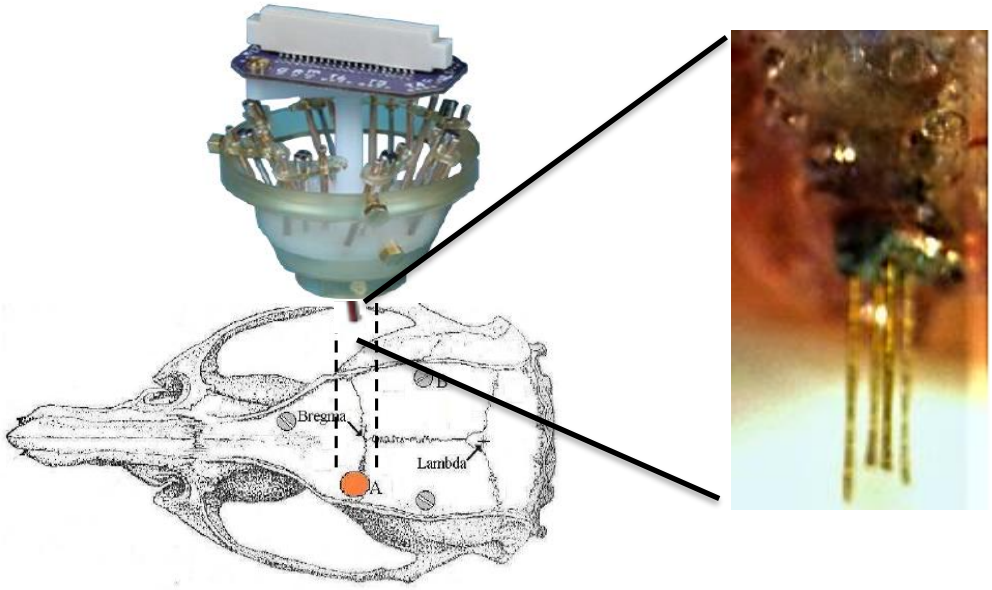


FIGURE Nº5. Scheme of craniotomy of rat for implanting microdrive into the pIC. Array of six tetrode implanted into the pIC (coordinates from Swanson's atlas: Bregma, -0.51 mm, midline, + 6.0 mm, and -6.5 mm from the cranial surface. Swanson, L.W. 1998). The magnification shows tetrodes out of the bundle. Tetrodes were constructed with four nickel-chrome (17 µm) wires twisted together.

Data acquisition, analysis and sorting of neuronal units. Extracellular signals action potentials are amplified approximately 15.000 times, with a bandpass filter of 600-6000 Hz, and transmitted to a Data Acquisition System Cheetah (Neuralynx, FIGURE Nº6). The signals are digitized at 32 KHz and stored on a pc for analysis offline. Electrophysiological signals were analyzed using MClust (A.D. Redish, University of Minneapolis, USA) that classify them according to their shape into individual clusters. Clusters formed by electrophysiological signals were further analyzed using the custom MATLAB script KlustaKwik (Kadir et al. 2014) that allows the manual selection of clusters with action potential shape signals. These signals are plotted in terms of relative amplitude (among other parameters) in different channels from the tetrode, with signals with similar characteristics forming an individual cluster. Signals were also plotted in the time domain to analyze their stability throughout the experiment. Next, waveforms in the 4 channels of the tetrode were analyzed again to confirm their action potential shape. Time interval between action potentials (inter spike interval, ISI) was also calculated. Neurons with more than 1% of action potentials with ISI less than 2 ms were excluded from the analysis, as they do not likely represent signals from one individual neuron.

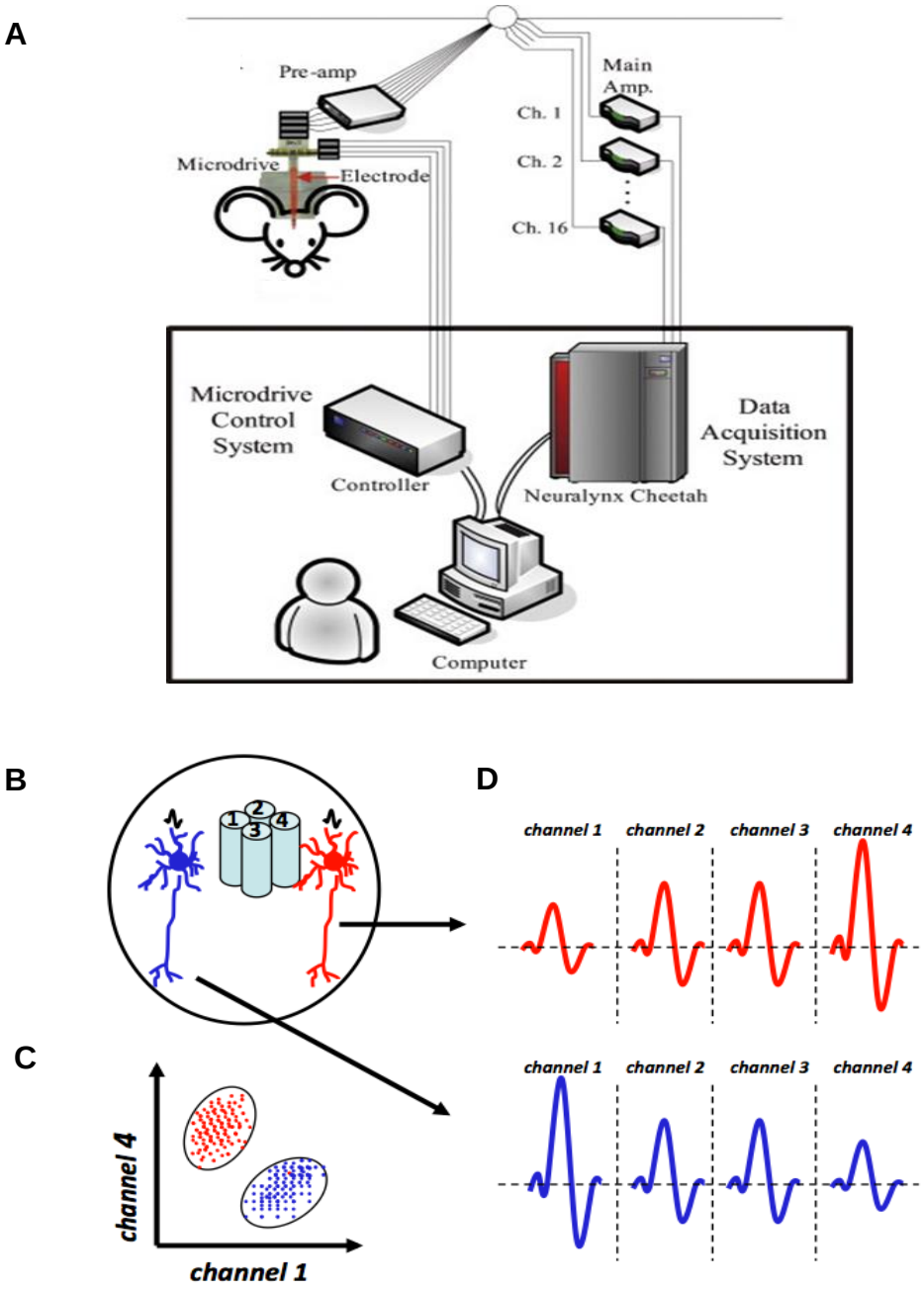


FIGURE Nº6. Representative scheme of single-units' isolation and identification. A. Showing schematic overview of the acquisition system. Adapted from Yang et. al, 2007. B. Representing the position of neurons. C. Dispersion plot showing energy of waveforms in channel 1 vs channel 4 of one tetrode. D. Average waveforms recorded in each channel of the tetrode.

Histology. After completing the experiments, the animals were deeply anesthetized with 7% chloral hydrate (350 mg/Kg; i.p.) 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, 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 3 alternate series of sections from each brain. One serie was stained with cresyl violet and other was used for immunohistochemistry. The sections were stained with Cresyl Violet and examined by light microscopy to determine cannula or tetrode placement. Rats with misplaced cannula/electrodes were discarded from the behavioral analysis. Rats implanted with tetrodes were previously anesthetized and electrolytic lesions were performed by applying anodic current of 25µA for 20s through two wires in each tetrode and the animal tail. They were killed 48 h later and were perfused as mentioned above.

5.2 Experimental design

Collar*.* The vinyl cat collars had a felt lined inner face to better keep the cat odor (dimensions: width 1.5 c, thickness 0.5, length 30 cm), one was worn by a domestic female cat for a week and the other was used as a control collar. The worn cat collar was kept in an air-tight plastic container and stored at 4 °C from one day (test day) to

3 days (trial 2 experiment 2). The behavioral sessions lasted 20 min, and the rats were exposed to the collar the last 10 minutes.

Cat Odor Test. We used a test chamber consisting of a clear Plexiglas rectangular chamber (60 × 40 × 40 cm, L, W, H). In one corner a cat collar was placed. Prior to experiments, rats lived under inverted 12/12 h light/dark cycles for 10 days (to conduct the behavioral tests during the rats' active phase) and during the last 3 days the rats were habituated to the test chamber during 30 min/day in the presence of an unworn cat collar. After habituation the rats were placed in the test chamber with the corresponding (worn or unworn) collar for 10 minutes (Test, day 0), and immediately after, the collar was replaced for another 10 minutes.

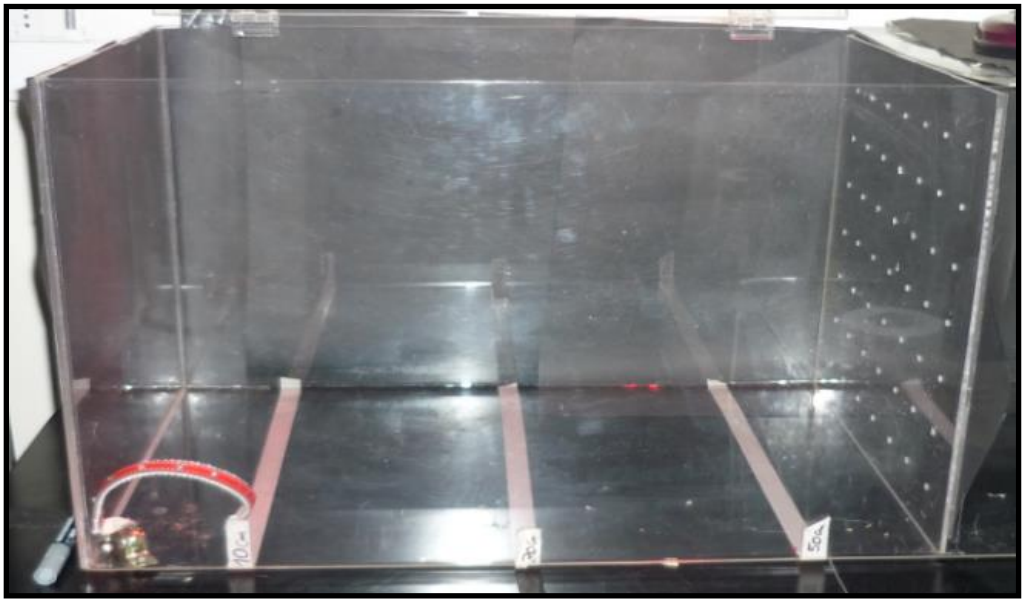


FIGURE Nº7. Test box and cat collar used for cat odor exposure test. Plexiglas rectangular chamber 60 cm length × 40 cm width × 40 cm height divided into three segments: near, middle, and far from the odor stimulus. In the corner of the right segment of the chamber a cat collar was located using a binder clip. The cat collar was made from acrylic wool (dimensions: width 15 mm, thickness 5 mm, length 300 mm).

Behavioral analysis. After each session, the chamber was cleaned with ethanol solution (5% v/v). All experiments were performed in a room illuminated by a red light bulb (80 watts) placed 20 cm above the chamber. Behaviors analyzed included: A) Freezing, defined as a complete absence of movements, except for respiration. B) Risk Assessment, a pattern of investigation of the threat source, including scanning with the head, flat back/stretch attend postures (Blanchard et al., 2005; Choy et al., 2012). C). Exploratory activity when the rat is engaged in free ambulatory behavior. D) Grooming, when the rat is engaged in cleaning itself. E) Contact with the stimulus, when the rat makes direct tactile contact with the collar, including chewing or the use of vibrissae (Dielenberg et al., 2001), see FIGURE Nº8. These behaviors were video-recorded using a horizontally mounted video camera placed 75 cm above and scored off-line by an experimenter blind to treatment.

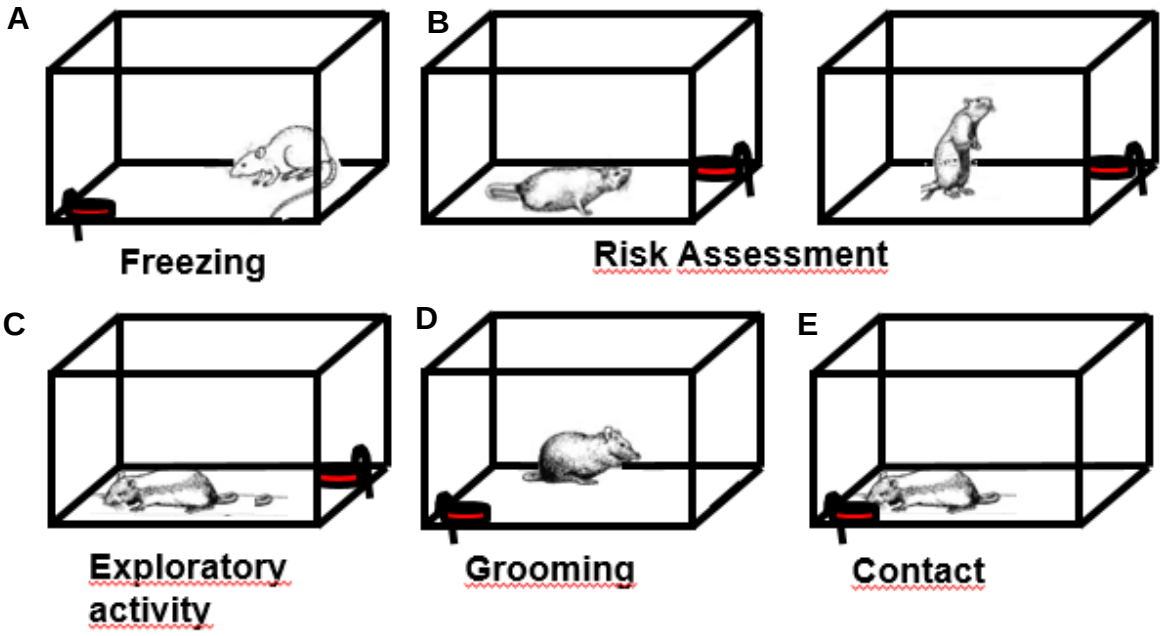


FIGURE Nº8. Representation of innate defensive and non-defensive behaviors. A-B Defensive behaviors such as freezing and risk assessment increase in response to threat. C-E. Non-defensive behaviors as exploratory activity, grooming and contact decrease or not in response to environmental threat.

5.3 Objective 1: Determine whether the inactivation of the pIC decreases innate defensive behaviors by pharmacological manipulations.

5.3.1 Experiment 1: *Evaluate the effect of short-term pIC inactivation on innate defensive behaviors using Muscimol, a GABAA-R agonist.*

To evaluate whether the pIC has a role in innate defensive behaviors, the rats were implanted with microinjection cannulae placed bilaterally in the pIC. Next day, the rats were individually housed and kept in an inverted cycle regime (see later in section 6.1.3). After habituation, we evaluated the effect of pIC inactivation on the innate fear response to cat odor (Cat Odor Test, day 0). The rats received Muscimol (0.5µg/µL 0.5µL/side) or saline (0.5µL/side) into the pIC or the somatosensory cortex (SS) and 30 minutes after the rats were placed in the test chamber with the unworn familiar collar (Control Collar) for 10 minutes, and immediately after replaced by the worn collar (Cat Odor Test) or not during another 10 minutes. Additionally, we used normal rats -distributed in two groups: Naïve No-Odor, Naïve Cat-Odor- to measure the effectiveness of cat odor stimulus, see section 6.1.2.

5.3.2*.* Experiment 2*: Evaluate the effect of a long-term pIC inactivation on defensive behaviors* using NSTX, a lasting (a week) voltage-gated sodium channel blocker, on repeated exposures to cat odor.

To evaluate the prolonged effect of pIC inactivation in the fear/anxiety experience, the rats were bilaterally implanted with microinjection cannulae in the pIC (surgery) and next day, the rats were individually housed and kept in an inverted cycle regime (see later in section 6.1.4). On Day 0, the rats were left in the test chamber for 10 minutes and then exposed to cat odor other 10 minutes and the behavioral performance was video-recorded and scored off-line (Cat Odor Test). Immediately after cat odor exposure, rats were injected into the pIC with NSTX or sterile saline under 2% Isoflurane anaesthesia. Twenty-four hours (ReTest) after the microinjection into the pIC, rats were returned to the chamber during 20 minutes and only the last 10 minutes were exposed to cat odor. This methodology was replicated on next day (ReTest 2), 4 days (ReTest 3), 14 days (ReTest 4) and 29 days (ReTest 5) after a single microinjection.

5.3.3 Experiment 3: *Evaluate the effect of pIC inactivation on context to cat odor using Muscimol, a GABAA-R agonist.*

To evaluate whether the pIC has a role in learned fear, the rats were implanted with microinjection cannulae placed bilaterally in the pIC. Next day, the rats were individually housed and kept in an inverted cycle regime (see later in section 6.1.5) After habituation, rats received Muscimol (0.5μg/μL 0.5μL/side) or saline (0.5μL/side) into the pIC, and 30 minutes after the rats were placed in the test chamber with a Control Collar (unworn) for 10 minutes. Immediately after control collar exposure, the collar was replaced for another one impregnated with cat odor for 10 minutes (Cat Odor Test, day 0). Next day, rats received saline or Muscimol and 30 minutes after were exposed to the context for 10 minutes, containing the control collar (Context, day 1). After that, the rats were re-exposed to cat odor for 10 minutes, to evaluate the changes during the second exposure (ReTest, day 1).

Data Analysis. Tests were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). All data are expressed as mean of percentage of total time spent in a behavioral state ± SEM and number of episodes of behavioral state ± SEM. Significant differences ($p<0.05$) were assessed with paired t-test, one-way ANOVA or two-way ANOVA followed by Tukey's *post hoc or* tests Bonferroni *post hoc* tests when appropriate.

5.4 Objective 2: Determine the pattern of activity of the pIC and other relevant brain regions during cat odor using the expression of c-fos.

5.4.1-3 *Determine the Fos immunoreactivity of the pIC during cat odor exposures, evaluate whether the retest modifies the Fos immunoreactivity of the IC and determine whether the Fos immunoreactivity of the pIC correlates with defensive behaviors.*

To evaluate the neuronal activity of the IC evoked by cat odor, we assessed the expression of c-fos, used as early marker of neuronal activity. Additionally, we evaluated whether the second exposure to cat odor modifies Fos activity. On day 0 (first exposure to cat odor), the rats were exposed to unworn collar for 10 minutes and immediately after, to cat odor collar for another 10 minutes, while the behavioral responses were recorded (Cat Odor Test). Another group was again exposed to cat odor on next day for 10 min after control collar presentation (ReTest). A control group was exposed to collar for 10 minutes and then 10 minutes with the same collar (Control Collar). Ninety min after the end of the cat odor exposure test, rats were killed and processed for measuring Fos immunoreactivity, see section 6.2)

Data Analysis. All statistical tests were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). The immunoreactivity was measured by c-Fos expression. All data are expressed as mean Fos-ir neurons ± SEM for each mm^2. Data were analyzed using Two-way ANOVA using level of bregma as

one factor and odor as the second factor followed by Bonferroni multiple comparisons. Furthermore, Spearman rank order correlations were performed to examine the relationship between neuronal activity and freezing behavior and the neuronal activity of subcortical nuclei and IC. All statistical assessments were considered significant when $p<0.05$.

5.5 Objective 3: Determine the pattern of activity of the pIC using electrophysiolgical recordings.

5.5.1 *Evaluate the single-unit activity in the pIC underlying innate defensive behaviors.*

To evaluate electrical activity in rats exposed to predator odor, the rats were implanted with a microdrive and then left in their homecages for promoting recovery. Recordings were performed during the night (after 21:00 h) in a dark room, during rat's active phase. Two hours before starting the experiment, the rat was connected to the recording system to search for unit activity, by gently lowering tetrodes in 40 µm steps (160 µm for each total revolution of the drive screw) until stable signals were acquired in most tetrodes. Later, the rat was placed in the test chamber with the control collar for 10 minutes, and immediately after the collar was replaced by the one with cat odor for another 10 minutes (Cat Odor Test, see section 6.3.1).

5.5.1.1 Behavioral analysis.

Time spent in behaviors during test was scored offline. Behavioral expression of innate fear was quantified as “freezing episodes”, defined as the absence of movement except for breathing (Blanchard and Blanchard 1969) and innate anxiety by measures of “risk assessment episodes” as observatory, side to side head movements without locomotion for at least 1s (Choy et al., 2012) for both. Data were expressed as percentage of time spent in behavior ± SEM and number of episodes ± SEM during the test. Differences for each behavior were analyzed with paired t-test.

5.5.1.2 Spike data analysis.

For characterization of recorded neurons, two components were evaluated: we calculated the distribution of peak to trough duration and repolarization at 0.46 ms for each cell (Ardid et al., 2015). The peak to trough duration was defined as the time interval from minimum to its maximum and the time for repolarization was defined as time at which the waveform decayed 25% from its peak value.

To detect changes in the activity of each neuron in response to cat odor, we calculated the FR in 2 s bins along the test (10 minutes’ cat odor exposure plus 2 minutes before cat odor, FIGURE Nº8) and calculated the mean FR from the bins inter-behavior-interval (IBI) and the mean FR from the bins during each freezing and obtained the difference between them. Then, we randomly mixed the bins across the

entire recording time and calculate a new mean FR difference between the surrogate before and the surrogate after bins (Maris and Oostenveld, 2007). We repeated this permutation procedure 1000 times and obtained a distribution of all the mean FR differences between before and after surrogate periods. From this distribution, we obtained a value representing the 5% limit of the largest differences and employed this value as the significant limit for the original mean FR difference for each neuron. Neurons were classified as associated with freezing when their FRs changed significantly only during freezing behavior. If activity changed during IBI and the activity persists during behavior, neurons were classified with a generalized response. A Fisher exact test was used to detect differences in the proportion of neurons responsive during freezing.

To better show the magnitude of the changes in the FR (not its significance, which was analyzed using the permutation test describe above). We considered from 6 s before to 6 s after freezing onset and stacked all freezing behaviors to obtain an averaged FR. Next; we calculated an averaged FR and a standard deviation for the first 3 bins (baseline).

6. RESULTS

6.1 Objective 1. Determine whether the inactivation of the pIC decreases innate defensive behaviors by pharmacological manipulations.

6.1.1 *Histological analysis.*

In order to evaluate whether the cannulas implanted were in the correct position along the pIC, from levels +0.95 to -2.45 mm from bregma; at the end of the behavioral experiments the animals were transcardially perfused and their brains were processed for cresyl violet staining and then analyzed under light microscopy.

This analysis (FIGURE Nº9) revealed 43 rats that showed a successful cannulation and received local microinjections into the pIC (Sal-pIC $_{exp\ 1}$, n=7; Mus-pIC $_{exp1}$, n=7; Sal-pIC $_{exp\ 2}$, n=6; NSTX-pIC $_{exp\ 2}$, n=6; Sal-Sal $_{exp\ 3}$, n=6; Sal-Mus $_{exp\ 3}$, n=7; Mus-Sal$_{exp\ 3}$, n=4) showing a major density of cannula tips between -0.51 to -0.82 from bregma.

Ten additional rats received bilateral injection into the SS (Muscimol; n=5 and NSTX: n=5 for experiments 1 and 2 respectively), exhibiting the injection cannula tip between levels 0 and -1.52 mm relative to bregma.

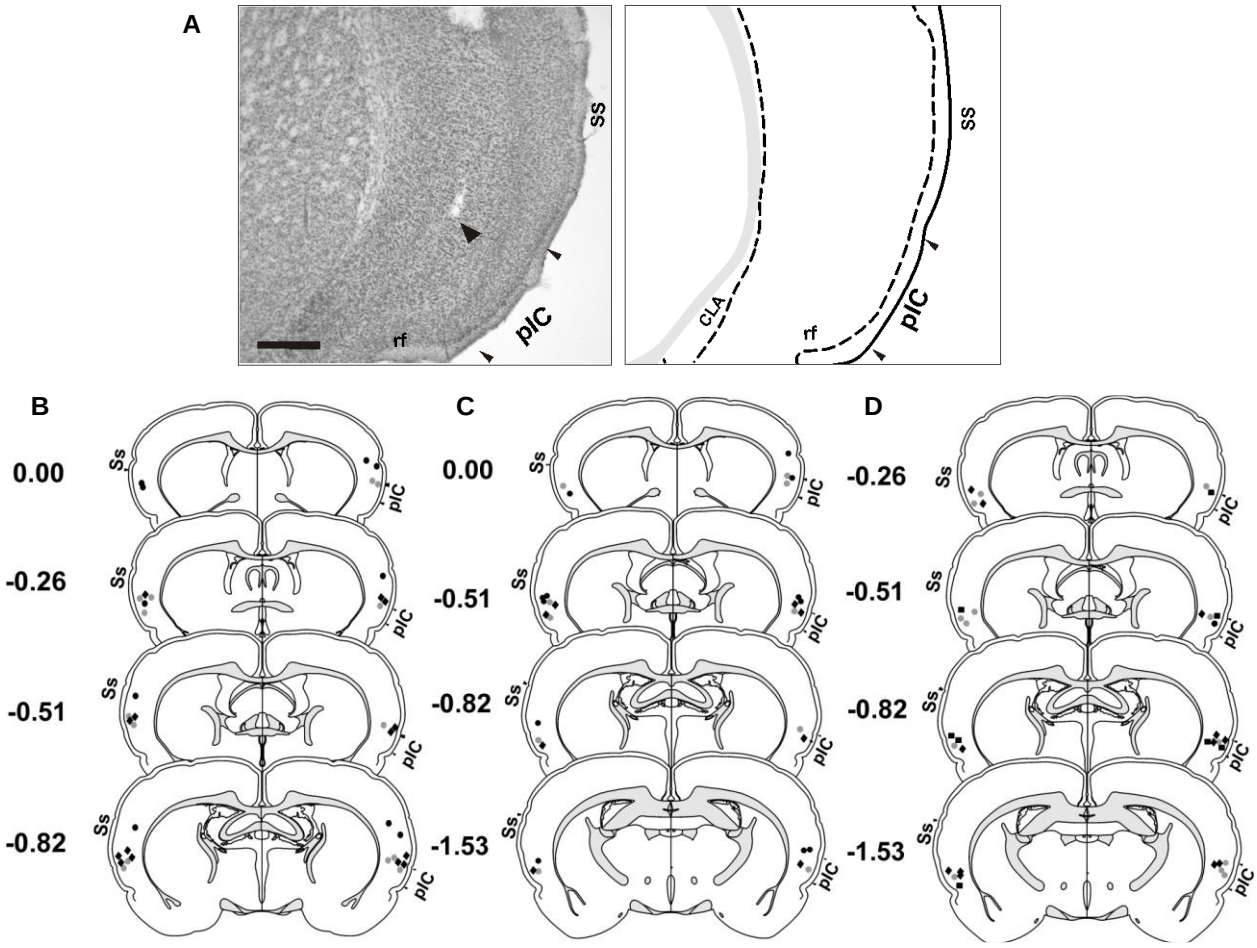


FIGURE Nº9. Representative example of the anatomical placement of the injection cannula tips in the posterior insular cortex (pIC). A. Left panel, photomicrograph of a Nissl stained coronal section showing the endpoints of the injection cannula tip (arrowhead) in the pIC and a schematic drawing (right) modified from Swanson's atlas. B, C and D. Reconstructions of the different injection sites of saline and Muscimol into the pIC (black squares and gray circles circles, respectively) and Muscimol into SS (black circles). Injection sites of saline and NSTX into the pIC (black squares and gray circles) and NSTX into the SS (black circles) for Experiment 2. Injection sites of saline and Muscimol into the pIC (black squares and gray circles, respectively) for Experiment 3. Scale bar indicates 500 µm. Abbreviations: CLA, claustrum; rf, rhinal fissure; SS, somatosensory cortex (Swanson, 1998).

6.1.2 Acute exposure to cat odor induces an increase of innate defensive behaviors.

It is known that predator odor test elicits defensive behaviors, which reflect both fear and anxiety in rodents. Commonly, fear is measured as flight, avoidance or freezing behaviors and anxiety is measured as stretch postures and vigilant scanning, broadly known as risk assessment behaviors (Blanchard et al., 2001; Dielenberg and McGregor, 2001; Apfelbach et al., 2005; Takahashi et al., 2005). Here, we performed a test chamber that helped us to reduce the extensive behavioral repertoire and amplifies the response to cat odor (Dielenberg et al., 2004). The rats were unable to escape and hide from the cat odor, hence, they exhibited a robust freezing and vigilant scanning in response to cat odor exposure.

FIGURE Nº10A shows the experimental protocol; on test day, normal rats were exposed to control collar during the first ten minutes. Rats expressed a variety of behaviors that changed after the introduction of the cat collar impregnated with cat odor; see methods, section 5.3.1). We quantified freezing, risk assessment as a measure of defensive behaviors and exploratory activity, grooming and contact with the stimulus as non-defensive behaviors (FIGURES Nº10 and11, respectively).

The rats showed low freezing and risk assessment behaviors in response to control collar (FIGURE Nº10), while exploratory activity, grooming behavior and contact with the stimulus remained relatively high (FIGURE Nº11). Once the cat collar was replaced by, rats showed an increase in defensive behaviors both freezing (FIGURE Nº10B) and risk assessment behaviors (FIGURE Nº10C).

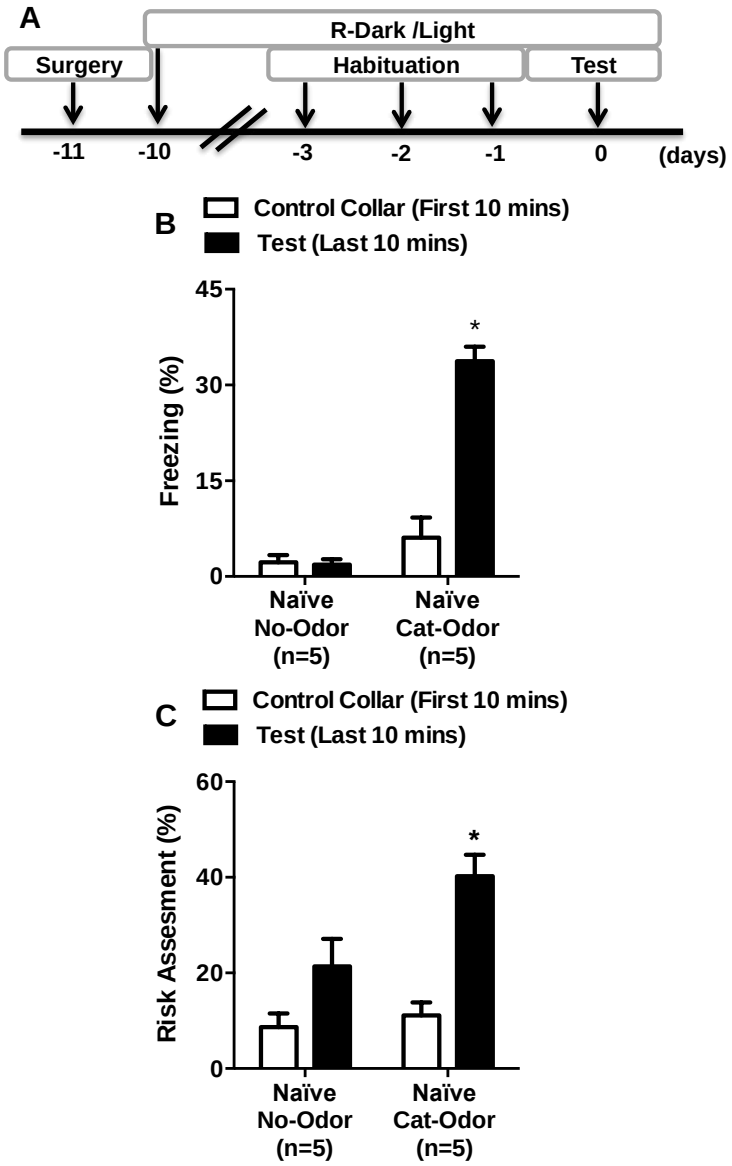


FIGURE Nº10. Expression of innate defensive behaviors in rats exposed to cat odor. A. Timeline of experimental design. During test day, normal rats were exposed to control collar (unworn familiar collar) during the first ten minutes and the next ten minutes exposed to a collar with cat odor (Naïve Cat-Odor, n=5). Another group was exposure for second time to a control collar (Naïve No-Odor, n=5). B-C. The bars show the percentage of time spent in each defensive behavior: freezing and risk assessment. *p<0.05; t-test. All values are means +SEM.

In contrast rats showed a decrease in exploratory activity (FIGURE Nº11A) and grooming behavior (FIGURE Nº11B). Contact with the stimulus (FIGURE Nº11C) did not change. The rats exposed to control collar (Naïve No-Odor group) during the cat odor phase of the test showed higher levels of exploratory activity and grooming compared to those exposed to cat odor (FIGURES Nº11A and B). We also observed a tendency to increase the expression of risk assessment but did not show significant difference. In summary, these data showed that high freezing levels were displayed only in the presence of cat odor (FIGURE Nº10B), confirming this paradigm useful to study innate fear and anxiety in rats.

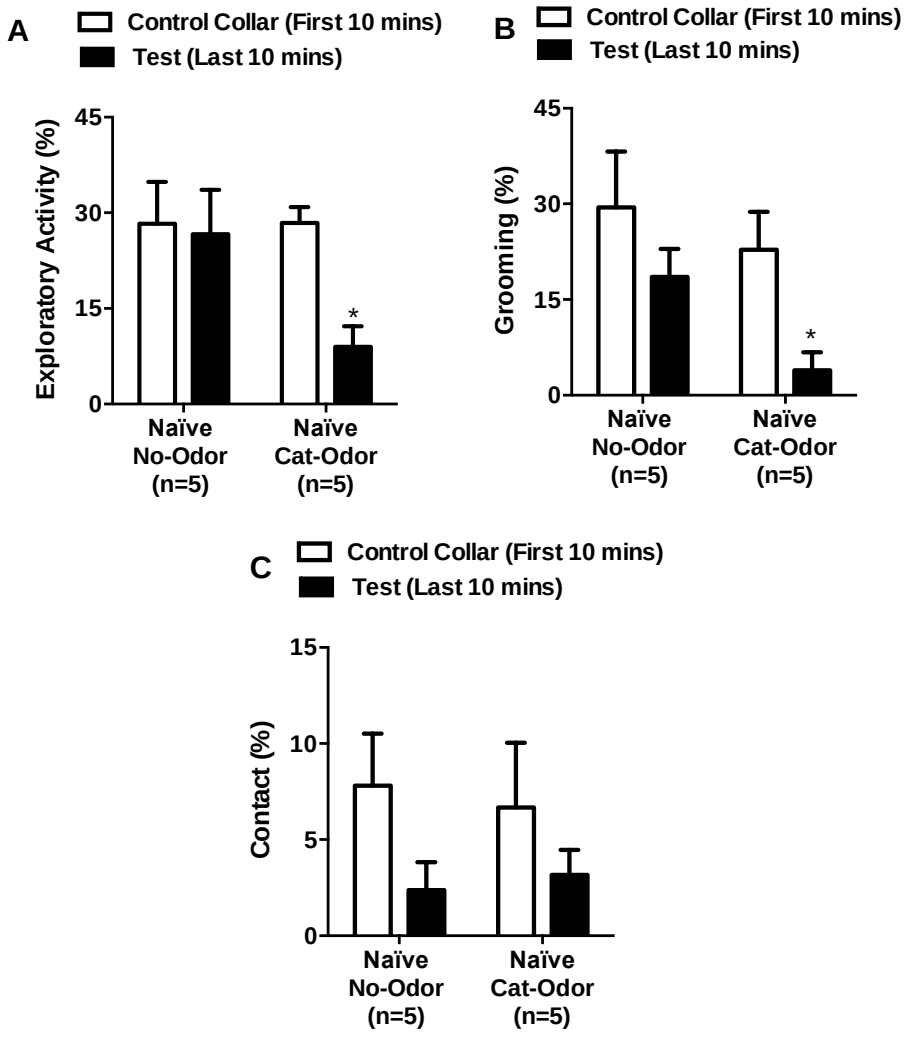


FIGURE Nº11. Expression of innate non-defensive behaviors in rats exposed to cat odor. A-C. The bars show the percentage of time spent in each non-defensive behavior: exploratory activity, grooming and contact. *p<0.05; t-test. All values are means +SEM.

6.1.3 Short-term pIC inactivation reduces freezing behavior as measure of innate fear expression.

This experiment was aimed to evaluate whether the activity of the pIC is necessary to display innate defensive behaviors. We assessed the effect of pIC inactivation by a single bilateral microinjection of Muscimol 30 minutes before test (Fig 10A, see methods section 5.3.1). The rats that received Muscimol infusion into the pIC showed no differences in the freezing response relative to control groups during the control collar (*post hoc* comparison between Sal-pIC and Mus-SS groups; $p>0.05$). However, when these rats were exposed to cat odor they showed no increase in freezing (FIGURE Nº12A). A Two-way ANOVA of freezing behavior revealed a main effect of Muscimol ($F_{(4,50)} =6.667$, $p<0.001$), of odor ($F_{(1,50)} =30.20$, $p<0.0001$) and a significant interaction ($F_{(4,50)} =6.413$, $p<0.001$). *Post hoc* comparisons confirmed that Sal-pIC and Mus-SS rats showed a significant increase in freezing when exposed to cat odor, compared to control collar ($p<0.01$; $p<0.001$, respectively).

Risk assessment increased under cat odor (FIGURE Nº12B). A Two-way ANOVA of risk assessment behaviors showed a main effect of odor ($F_{(1,50)} = 23.78$; $p< 0.0001$), but no effect of Muscimol nor interaction of odor with treatment. *Post hoc* comparisons showed significant changes in risk assessment in Sal-pIC and Mus-SS rats when exposed to cat odor, compared to the control collar ($p< 0.05$).

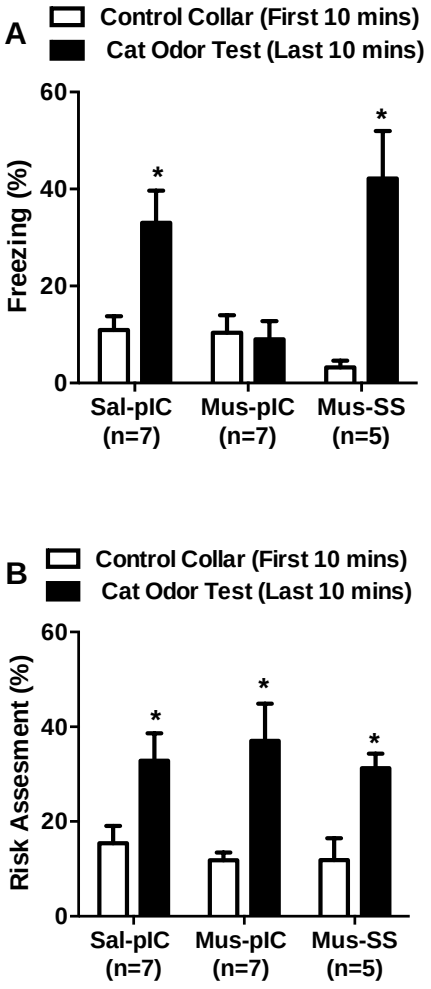


FIGURE Nº12. Inactivation of pIC prior to first exposure to cat odor abolished the innate fear behavior. Rats received Saline (n=7) or Muscimol (n=7) into the pIC or Muscimol into the SS (n=5) thirty minutes before test: the first ten minutes in response to control collar and the next ten minutes exposed to a collar with cat odor. A and B. The bars show the percentage of time spent in each defensive behavior: freezing and risk assessment. Note that Muscimol-pIC rats displayed a reduction of freezing behavior (fear) in the presence of cat odor relative to Saline-pIC and Muscimol-SS rats. In contrast, risk assessment behavior (anxiety) was not altered by the infusion of Muscimol into the pIC. *p<0.05, **p<0.01; Two-way ANOVA followed by Bonferroni test. All values are means +SEM.

Additionally, we found an effect the odor on non-defensive behaviors: the exploratory activity (FIGURE Nº13A) and grooming behavior (FIGURE Nº13B) decreased in response to cat odor. A Two-way ANOVA of exploratory activity revealed a significant effect of odor ($F_{(1,50)} = 28.86$, $p<0.0001$) but no effect of Muscimol nor interaction. *Post hoc* comparisons showed that saline-pIC and Muscimol-pIC rats showed a significant decrease in exploratory activity compared when those rats were exposed to control collar ($p<0.05$). The fraction of time spent in grooming was reduced by exposure to cat odor (FIGURE Nº13B). A Two-way ANOVA of grooming, revealed a main effect of odor ($F_{(1,50)} = 38.27$; $p<0.0001$) but not of Muscimol and interaction. *Post hoc* comparisons confirmed that Sal-pIC, Mus-pIC and Mus-SS rats showed less grooming than control rats (no odor, $p<0.05$) in cat odor condition, and their pre-odor condition ($p<0.05$). *Post hoc* comparisons revealed that Mus-SS rats had a higher percentage of grooming relative to naïve ($p<0.05$), Sal-pIC rats ($p<0.01$) and Mus-pIC rats ($p<0.05$) during the control collar exposure. Finally, we did not find significant changes along the test in the time spent in contact with the stimulus (FIGURE Nº13C). A Two-way ANOVA of contact with the stimulus revealed no effect of odor, treatment or interaction.

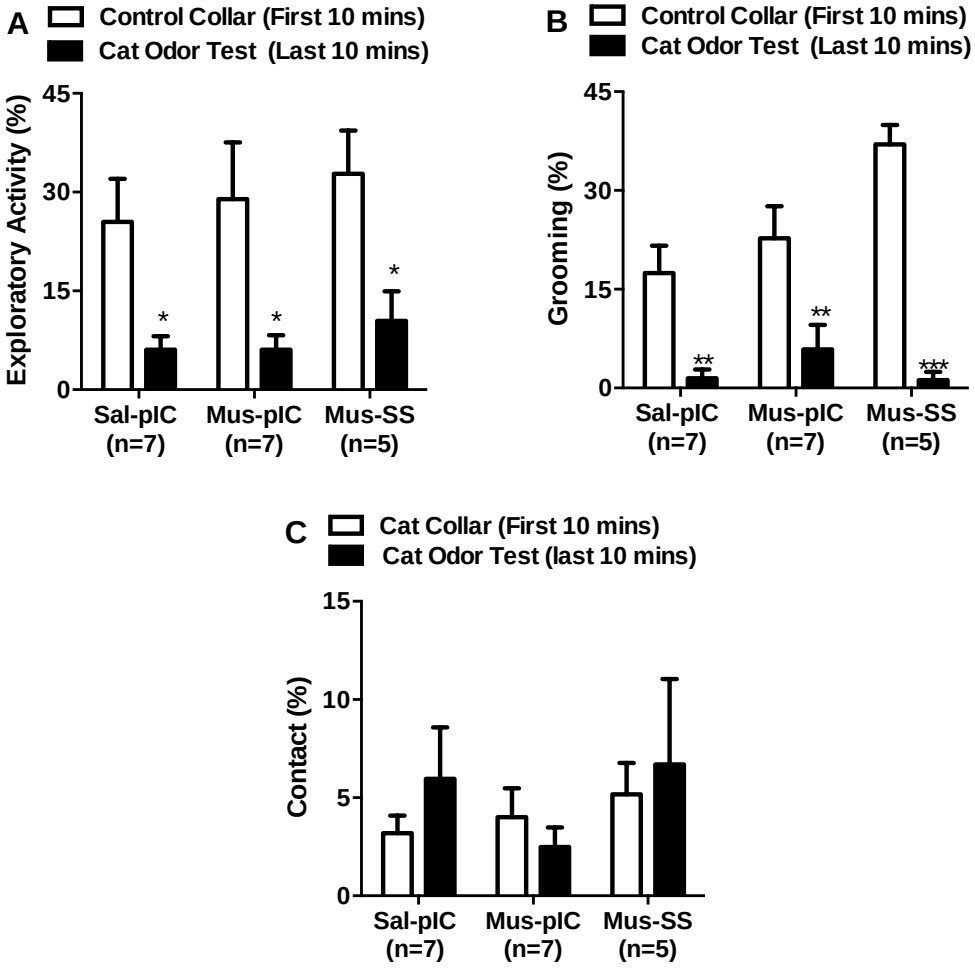


FIGURE Nº 13. Inactivation of pIC prior to first exposure to cat odor had no effect on non-defensive behaviors. Rats received Saline (n=7) or Muscimol (n=7) into the pIC or Muscimol into the SS (n=5) thirty minutes before test: the first ten minutes in response to control collar and the next ten minutes exposed to a collar with cat odor. A-C. The bars show the percentage of time spent in each behavior: exploratory activity, grooming or contact behaviors. *p<0.05, **p<0.01, ***p<0.001; Two-way ANOVA followed by Bonferroni test. All values are means +SEM.

These results indicate that the Muscimol inactivation of the pIC impaired the expression of unconditioned freezing, but not risk assessment and non-defensive behaviors in response to acute exposure to cat odor. It has been shown that IC is implicated in contextual and auditory fear learning (Alves et al., 2013; Casanova et al., 2016). Thus, we next decided to assess whether pIC is involved in learned responses to cat odor.

6.1.4 Long-term pIC inactivation reduces fear response in response to consecutive cat odor exposures.

Since short-term pIC inactivation had only an effect on innate fear expression, we studied whether fear expression is affected during a prolonged inactivation of the pIC induced by repeated cat odor exposures (FIGURE Nº14 and see methods section 5.3.2). We assessed the prolonged effect of pIC inactivation on repeated exposures to cat odor, using an extended pIC inactivation induced by a single bilateral microinjection of NSTX after the first exposure to cat odor. We re-tested these rats the next day (ReTest), subsequent day (ReTest 2), four days (ReTest 3), fourteen days (ReTest 4) and twenty-nine days (ReTest 5) after microinjection (FIGURE Nº14A), each time exposing the rats to cat odor.

All groups (NSTX-pIC, NSTX-SS and Sal-pIC rats) showed no differences in behavior before pIC inactivation, as described in Experiment 1. These rat groups spent the experiment time on defensive behaviors: 29% in freezing (One-way ANOVA, $F_{(2, 14)} = 0.3002$, $p>0.05$) and 35% in risk assessment behaviors (One-way ANOVA, $F_{(2, 14)}$

= 3.227, p>0.05), see Cat Odor Test in FIGURE Nº14. The rest of the time the rats displayed behaviors not related to the test.

In subsequent cat odor tests, we found that NSTX inactivation of the pIC resulted in a long-term reduction of freezing (FIGURE Nº14B). A Two-way ANOVA of freezing behavior revealed a main effect of NSTX treatment ($F_{(2,84)} = 12.51$, $p<0.0001$), time ($F_{(5,84)} = 10.03$, $p<0.0001$) but not an interaction ($F_{(10,84)} = 1.15$, $p>0.05$). *Post hoc* comparisons confirmed that NSTX-pIC rats showed significantly less freezing levels than Sal-pIC or NSTX-SS rats on ReTest ($p<0.001$), ReTest 2 ($p<0.001$) and ReTest 3 ($p<0.01$) compared to Sal-pIC rats. In contrast, we did not observe significant differences between NSTX-pIC rats and their ReTests 4 or 5 ($p>0.05$). Additionally, we observed an effect of NSTX on SS during ReTests 2 and 3 ($p<0.05$) compared to Sal-pIC rats, but not in the remaining ReTests ($p>0.05$). We also noted that repeated exposure to cat odor showed habituation between ReTest 4 and ReTest 5 (One-way ANOVA with repeated measures, $F_{(5, 25)} = 8.73$, $p<0.0001$). *Post hoc* comparisons showed that ReTests 4 and 5 had significantly less freezing than Cat Odor test, ReTest and ReTest 2. *Post hoc* comparisons revealed that Cat Odor Test > ReTest 4 ($p>0.05$); Cat Odor Test > ReTest 5 ($p>0.01$); ReTest 1> ReTest 4 ($p>0.05$); ReTest 1> ReTest 5, $p<0.05$); ReTest 2 > ReTest 4, $p<0.05$); ReTest 2 > ReTest 5, $p<0.01$) demonstrating that the levels of freezing during ReTest 4 and 5 were lower that during Cat Odor Test, ReTest and ReTest 2.

In addition, we found a significant reduction of risk assessment behavior in NSTX-pIC rats compared to Sal-pIC and NSTX-SS rats only during ReTest (FIGURE

Nº14C). A Two-way ANOVA of risk assessment behavior revealed a main effect of treatment ($F_{(2,84)}$ =12.91 $p<0.0001$) but no effect of time ($F_{(5,84)}$ =0.39, p=0.849) or interaction ($F_{(10,84)}$ =0.50, p=0.882). *Post hoc* comparisons confirmed that NSTX-pIC rats showed significantly more risk assessment than Sal-pIC rats ($p<0.01$) on ReTest, and had no significant effect on ReTest 2 ($p>0.05$) and ReTest 3 ($p>0.05$) after NSTX microinjection. We observed significant differences between NSTX-pIC and NSTX-SS rats on ReTest ($p<0.01$) and ReTest 2 ($p<0.01$), but not significant differences on the remaining cat odor exposures.

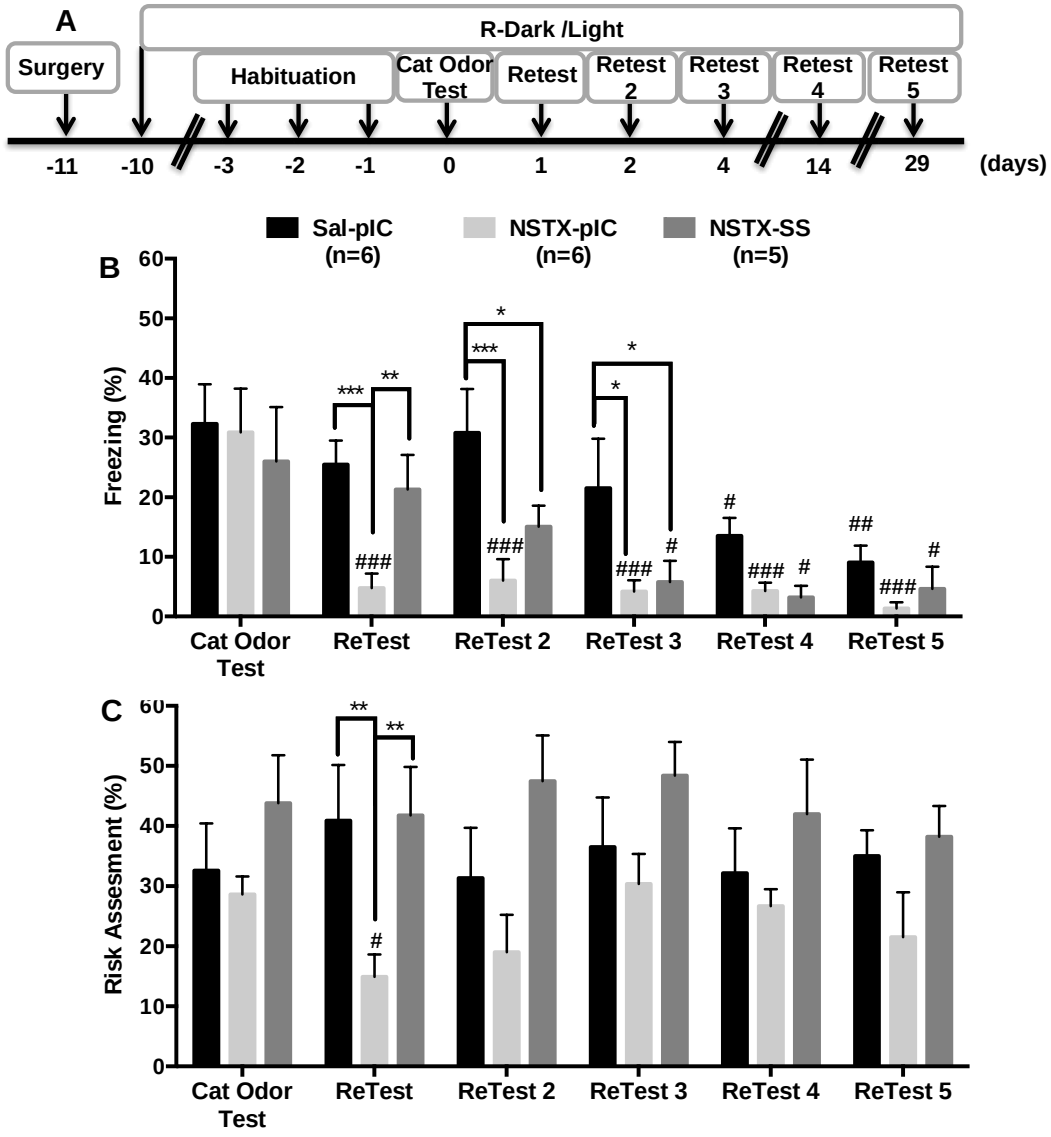


FIGURE Nº14. Long-term pIC inactivation induced a prolonged reduction in the expression of fear and a brief reduction of anxiety. A. Timeline of the experimental design. Immediately after first exposure to cat odor, rats were infused once with saline or Neosaxitoxin (NSTX) into the pIC. Another group was infused once with NSTX into the SS. In the subsequent tests (ReTest [day 1], ReTest 2[day 2], ReTest 3[day 4], ReTest 4 [day14] and ReTest 5 [day 29]) they were re-exposed to cat odor and the behavioral responses recorded. B-C. The bars show the time spent in freezing and risk assessment. *p<0.05, **p<0.01, ***p<0.001; comparisons between groups. #p<0.05, ##p<0.01 ###p<0.001; comparisons intra-groups relative to cat odor test (day 0). Two-way ANOVA followed by Bonferroni test. All values are expressed as mean + SEM.

Moreover, we also measured non-defensive behaviors displayed in response to cat odor along the experiment (SUPPLEMENTARY Nº1 and 2). The exploratory activity showed a significant increase in NSTX-pIC rats compared to Sal-pIC and NSTX-SS rats (SUPPLEMENTARY Nº 1). A Two-Way ANOVA of exploratory activity revealed a main effect of treatment ($F_{(2,84)}$ =11.59, p<0.0001), but no effect of time ($F_{(5,84)}$ =1.94, p>0.05) or interaction ($F_{(10,84)}$ =0.55, p>0.05). *Post hoc* comparisons confirmed that NSTX-pIC rats showed significantly more exploratory activity than Sal-pIC rats and NSTX-SS rats on ReTests 2 and 3 (p<0.05; p<0.01 and p<0.05 respectively). ReTest showed significant differences relative to NSTX-SS rats (p<0.05) but did not relative to Sal-pIC rats (p<0.05). ReTest 5 showed differences relative to Sal-pIC rats. For grooming behavior, a Two-way ANOVA for grooming revealed no effect of odor, treatment or interaction (SUPPLEMENTARY Nº 2A). Finally, the time spent in contact with the stimulus showed a significant increase relative to NSTX-SS rats a (SUPPLEMENTARY Nº 2B). Two-Way ANOVA of contact with the stimulus revealed a main effect of treatment ($F_{(2,84)}$ =6.71, p<0.01), but no effect of time ($F_{(5,84)}$ =1.60, p>0.05) or interaction ($F_{(10,84)}$ =0.59, p>0.05). *Post hoc* comparisons revealed more time spent in contacts in the NSTX-pIC compared to NSTX-SS on ReTest 5 (p<0.01).

Overall, these results show that the long-lasting pIC inactivation induced a prolonged reduction of the conditioned freezing and a short-term reduction of the risk assessment in response to chronic cat odor exposure, but no effect on non-defensive behaviors. These results confirm that the neuronal activity of the pIC is necessary for

expression of defensive behaviors, and suggest that pIC may be involved in the learning and encoding of fear memory.

6.1.5 Short-term pIC inactivation reduces learned defensive behaviors in response to context exposure.

Several studies have indicated that learning processes occur during cat odor cues exposure (Dielenberg and McGregor 1999; Dielenberg et al, 1999, Takahashi et al, 2008). It has been shown that rats that return to environment previously associated with predator context display defensive behaviors, cardiovascular effects and neuronal changes (Beck and Fibiger, 1995; Blanchard et al., 2001; Dielenberg et al., 2001) as well as during a second exposure to cat odor (ReTest, Staples et al., 2005). In order to assess whether the pIC has a role in learned defensive behaviors (context exposure), we transiently inactivated the pIC prior to test day 1, see methods, section 5.3.3, FIGURE Nº15A.

On day 0 (FIGURE Nº15) prior to experiments, rats received saline (Sal-Sal pIC and Sal-Mus pIC rats) or Muscimol (Mus-Sal pIC rats) into the pIC. During Cat Odor Test, Sal-Sal pIC and Sal-Mus pIC rats showed an increase on defensive behaviors: freezing (28% Sal-Sal $p<0.001$ and 27% Sal-Mus pIC $p<0.01$, FIGURE Nº 15B) and risk assessment (42% Sal-Sal $p<0.01$ and 35% Sal-Mus pIC $p<0.05$, FIGURE Nº 15C).

On day 1, Sal-Sal pIC rats showed high levels of freezing and risk assessment during re-exposure to the context and to the cat odor (26% and 40%, respectively;

FIGURE Nº15B and C, Context; 29% and 34%, respectively, FIGURE Nº15B and C, ReTest). However, rats that received saline into the pIC on day 0 and received Muscimol into the pIC 30 minutes before context (Sal-Mus pIC rats), showed low levels of freezing and risk assessment behaviors. A Two-way ANOVA of freezing behavior revealed effects in Muscimol ($F_{(2,56)}$ =6.9094, $p<0.01$), in context ($F_{(3,56)}$ =4.684, $p<0.01$) and interaction ($F_{(6,56)}$ =2.355, $p<0.05$). *Post hoc* comparisons confirmed that Sal-Mus pIC rats showed a significant decrease in conditioned freezing when they were re-exposed to the context and cat odor relative to the Sal-Sal pIC rats ($p<0.05$), but not Mus-Sal pIC groups ($p>0.05$).

For risk assessment (FIGURE Nº 15C), a Two-way ANOVA revealed a main effect of context ($F_{(3,56)}$=7.790, $p<0.001$). *Post hoc* comparisons confirmed that Sal-Mus pIC rats showed a significant decreased in risk assessment expression when rats were exposed to the context, compared to the cat odor test. This group also showed less risk assessment expression relative to Sal-Sal pIC rats ($p<0.05$) during context exposure. There was no difference between groups in risk assessment during Retest.

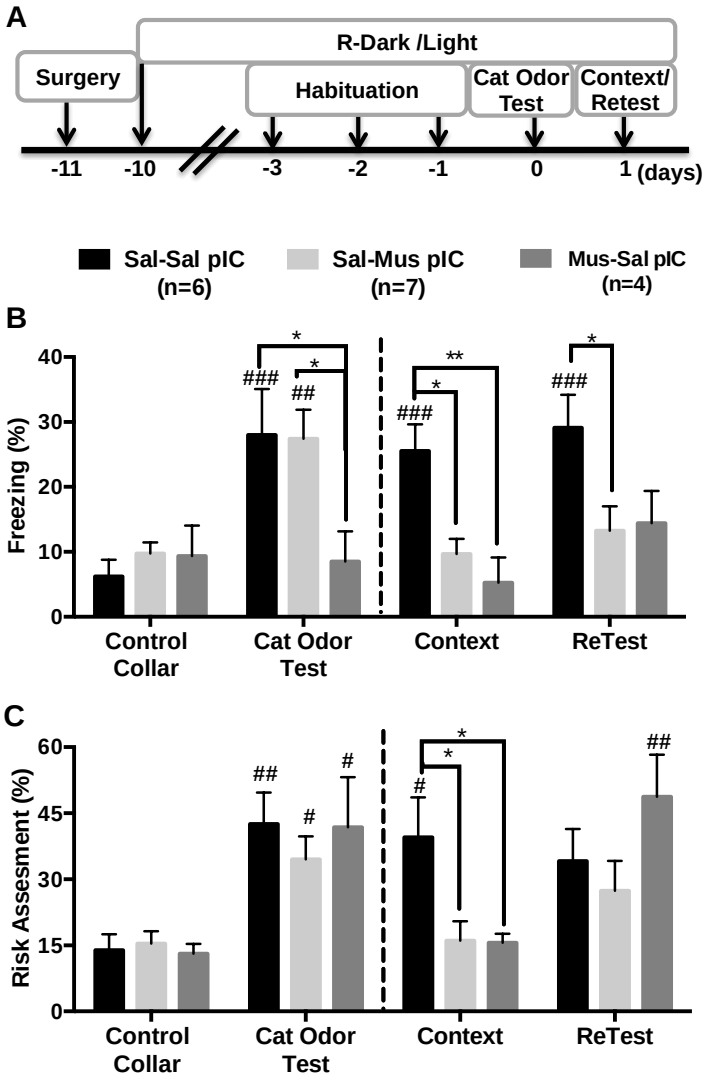


FIGURE Nº 15. Short-term pIC inactivation induces a decrease of defensive behaviors during context exposure. A. Timeline of the experimental design. Rats received Saline (n=13) or Muscimol (n=4) into the pIC thirty minutes before Test (Day 0). Next day, rats that had received saline previous day, they received Saline (n=6) or Muscimol (n=7) thirty minutes before context exposure. As well as rats that received Muscimol previous day, they received Saline (n=4) before context exposure. B-C. The bars show the percentage of time spent in each defensive behavior: freezing and risk assessment. *p<0.05, **p<0.01; comparisons between groups. #p<0.05, ##p<0.01 ###p<0.001; comparisons intra-groups relative to pre-odor (day 0). Two-way ANOVA followed by Bonferroni test. All values are expressed as mean + SEM.

Consistent with our previous results (see section 6.1.3), the pIC inactivation with Muscimol reduced the expression of freezing behavior, but not risk assessment (9% and 41% respectively; Mus-Sal pIC rats, FIGURE Nº15B and C, Cat Odor Test) in response to acute cat odor exposure relative to Sal-Sal and Sal-Mus groups. On day 1, these rats were injected with saline into the pIC and exhibited lower levels of both freezing and risk assessment during context exposure (5% and 15% respectively, $p<0.05$), and lower freezing, but normal levels of the risk assessment, during cat odor re-exposure (14% and 49% respectively, $p<0.05$, ReTest) relative to the other groups. Finally, we did not find an effect of Muscimol during context or retest sessions in exploratory activity, grooming and contact with the stimulus (SUPPLEMENTARY Nº3)

These results show that pIC inactivation abolished the context freezing and risk assessment, but the transient inactivation was not able to reduce the risk assessment behavior when rats were re-exposure to cat odor. These results strongly suggest that neuronal activity of the pIC is necessary for contextual fear conditioning.

6.2 Objective 2. Determine the pattern of activity of the pIC and other relevant brain regions during cat odor using the expression of c-fos.

6.2.1 *Neuronal activity of the pIC increases during cat odor exposure.*

Since the IC has been involved in emotions and our data of pIC inactivation show effects on behavioral responses to cat odor, we decided to study the effect of cat odor on the neuronal activity. Using Fos as marker of neuronal activation (Chaudhuri and Zangenehpour, 2002) we assessed the IC neuronal activity after cat odor exposure. A separate experimental group was sacrificed 90 min after exposed to the threatening stimulus (FIGURE Nº16).

We found a significant increase in neuronal activity of the pIC and the rostral agranular insular cortex (RAIC) in rats exposed to cat odor compared to rats exposed to a control collar; specifically, at levels +0.95 (t_{96}=2.773, p<0.01), -0.26 (t_{96}=2.867, p<0.05) and -1.08 (t_{96}=2.368, p<0.05, FIGURE Nº16B) from bregma to pIC and at levels +4.85 (t_{96}=3.428, p<0.001), +3.6 (t_{96}=2.181, p<0.05) and +2.8 (t_{96}=2.742, p<0.01, FIGURE Nº16B) from bregma to RAIC. Additionally, we measured neuronal activity in the IC of rats re-exposed to cat odor (ReTest, FIGURE Nº16C). We found a significant increase in the number of Fos-ir neurons in the IC of rats exposed to cat odor for a second period (ReTest, FIGURE Nº16C), both in the pIC, specifically at levels +0.95(t_{96}=2.257, p<0.05), +0.45 (t_{96}=3.406, p=0.001), -0.26 (t_{96}=2.481, p<0.05), -0.51 (t_{96}=2.173, p<0.05), -1.08 (t_{96}=2.453, p<0.05, FIGURE Nº16C), from bregma and RAIC,

specifically at levels +2,8 (t_{96}=2.033, $p<0.05$), +1,70 (t_{96}=2.187, $p<0.05$) and +1,20 (t_{96}=2.523, $p<0.05$, FIGURE Nº16C) from bregma.

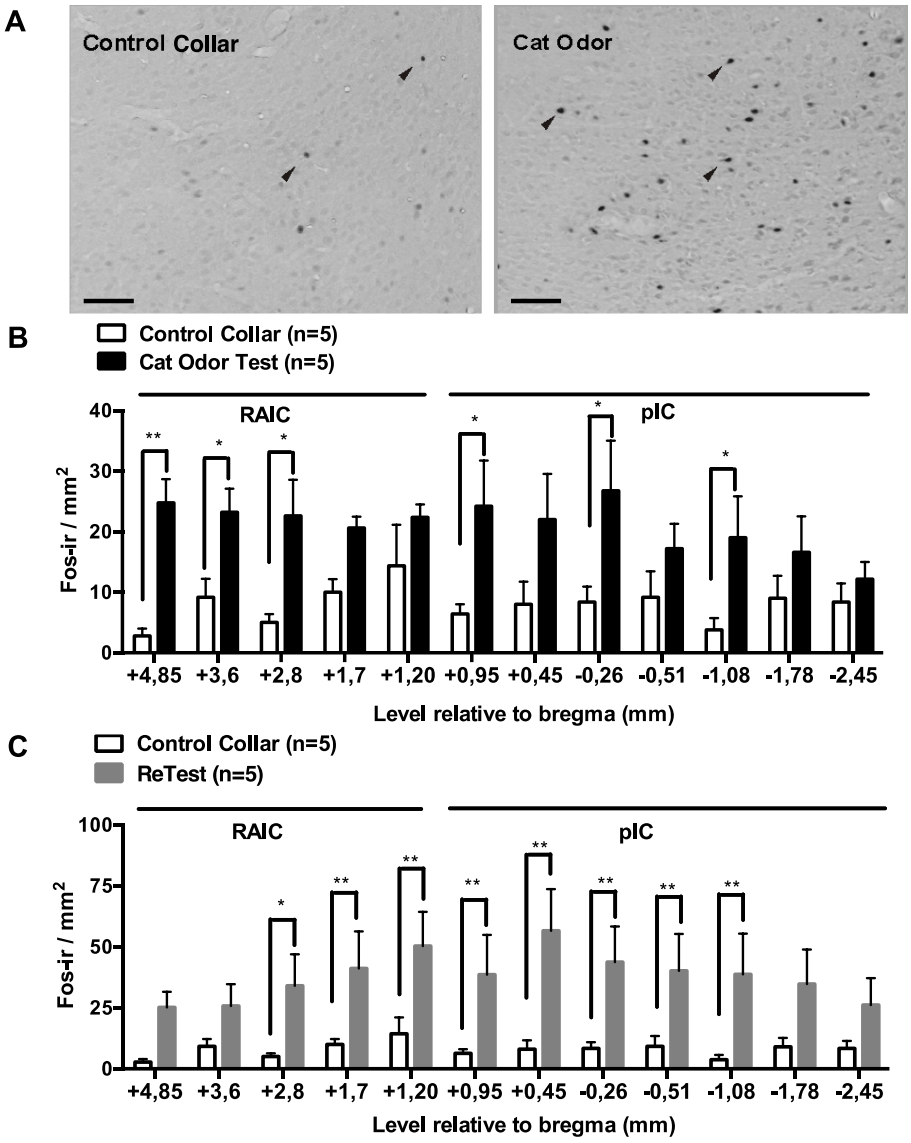


FIGURE Nº16. Innate fear was accompanied by an increase in the neuronal activity of the IC. A. Representative photomicrograph of the pIC showing near absence of positive Fos-ir in rats exposed to a control collar (left) and significant increase in number of positive Fos-ir in rats exposed to cat odor (right). Total quantification of Fos-ir in two different regions of the IC, the rostral agranular insular cortex (RAIC) and the granular field of the pIC. B-C. Quantification of Fos-ir at different levels from bregma of rats exposed once (B, Cat Odor Test) or twice to cat odor (C, ReTest). D. *p<0.05, **p<0.01, ***p<0.001; comparisons between groups. Two-way ANOVA followed by Bonferroni test. All values are expressed as mean + SEM of % total time of freezing expression. Scale bars indicate 200 µm.

These results indicate that neuronal activity of the IC increased either after the first or the second exposure to cat odor, and that this increased neuronal activity was accompanied by increased levels of fear expression.

6.2.2 *Neuronal activity of pIC neurons correlates with fear expression.*

To understand better the relationship between IC neuronal activity and fear expression we examined the correlation between freezing and the number of Fos-ir neurons in the IC during cat odor exposures. We observed high levels of freezing expression during the Cat Odor Test and ReTest (unpaired t- test, $p<0.05$ for both, FIGURE Nº17A) compared to the control collar. These rats showed a similar pattern of behavioral expression in both conditions (SUPPLEMENTARY Nº4 and 5A), comparable to our previous experiments.

We did not find a correlation between freezing behavior and neuronal activity induced by a single exposure to cat odor (Cat Odor Test). FIGURE Nº17B and C show the correlation analysis for the pIC activity (Spearman $r=0.41$, $p=0.25$) and the RAIC activity (Spearman $r=0.81$, $p=0.067$) during Cat Odor Test. In contrast, robust correlations between freezing behavior and the total number of Fos-ir neurons in the pIC (Spearman $r=0.98$, $p=0.0167$, FIGURE Nº17D) and the RAIC (Spearman $r=0.9$, $p=0.042$, FIGURE Nº17E) were observed during ReTest.

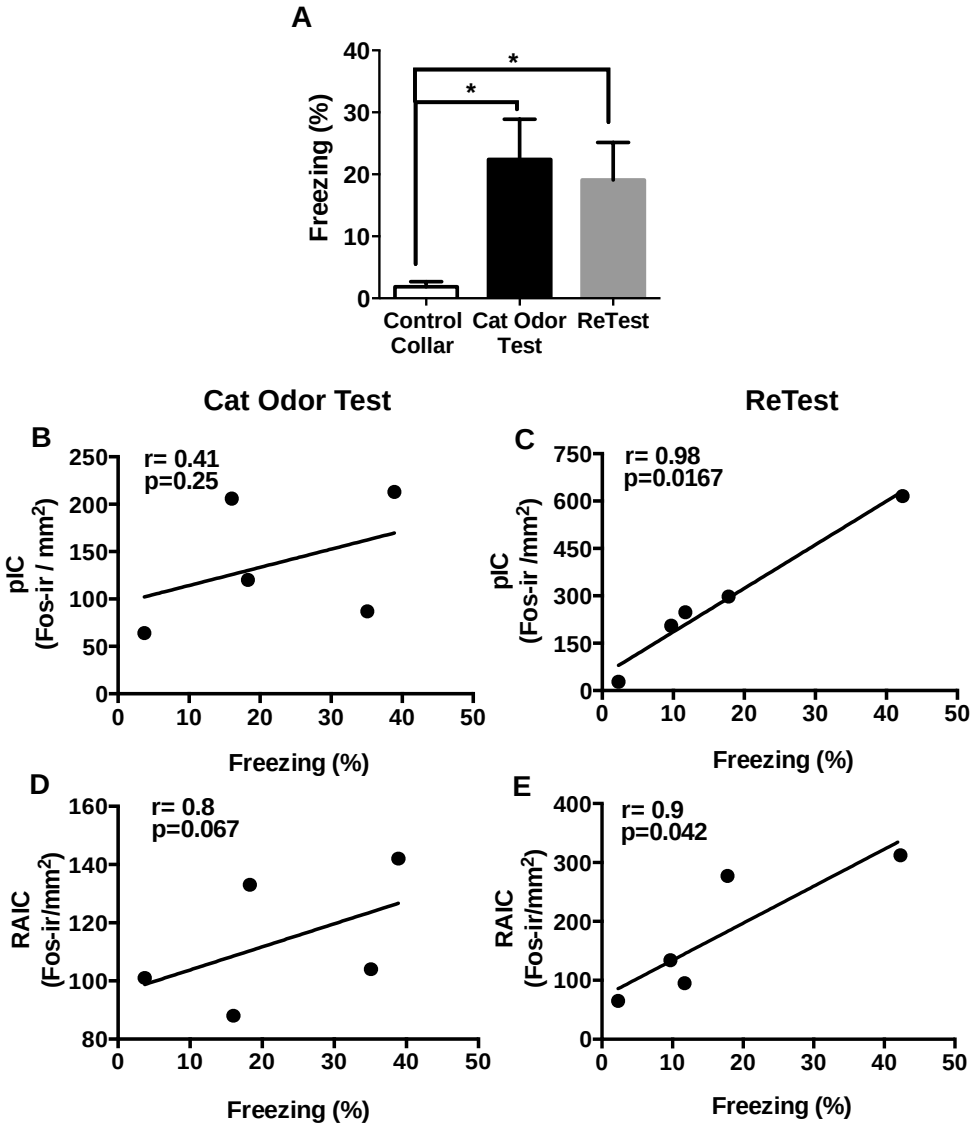


FIGURE Nº17. The neuronal activity of the IC is highly correlated with freezing behavior in response to second exposure to cat odor. A. Innate fear expression in response to cat odor exposure showed a higher level of freezing relative to rats exposed to the control collar (n=5) either on first (Cat Odor Test, n=5) or second exposure (ReTest, n=5). B-C. Correlations between % of freezing over total time and number of Fos-ir neurons in different IC regions: pIC and RAIC, respectively. D-E. Correlations between % of freezing over total time and number of Fos-ir neurons induced by a second exposure in the pIC and RAIC respectively. Correlation value and significance is showed in inset. *p<0.05. One-way analysis of variance followed by Tukey test for freezing (A) and Spearman rank order correlation (B-E).

Regarding to the significant differences in the activity of different levels of the pIC in presence of cat odor, either first or second exposure, and the activity elicited in response to control collar observed in FIGURES Nº16B and C, we found a significant correlation of Fos-ir neurons at the level -1.08 from bregma with freezing behavior in Cat Odor Test (Spearman r = 0.98, p =0.017, FIGURE Nº18A) and ReTest (Spearman r = 0.97, p =0.033, FIGURE Nº18B). We did not find a significant correlation between Fos expression in the IC and risk assessment (Cat Odor Test: Spearman r = -0.3, p =0.683 for pIC and Spearman r = -0.9, p =0.083 for RAIC; ReTest: Spearman r = -0.3, p =0.7; for pIC and Spearman r = -0.4, p =0.517 for RAIC). These data show that expression of freezing is positively correlated with an increase in pIC neuronal activity, suggesting a discrete area of the pIC, which may be relevant in the expression of freezing. Overall, our data support a role for the pIC on fear expression both innate and learned.

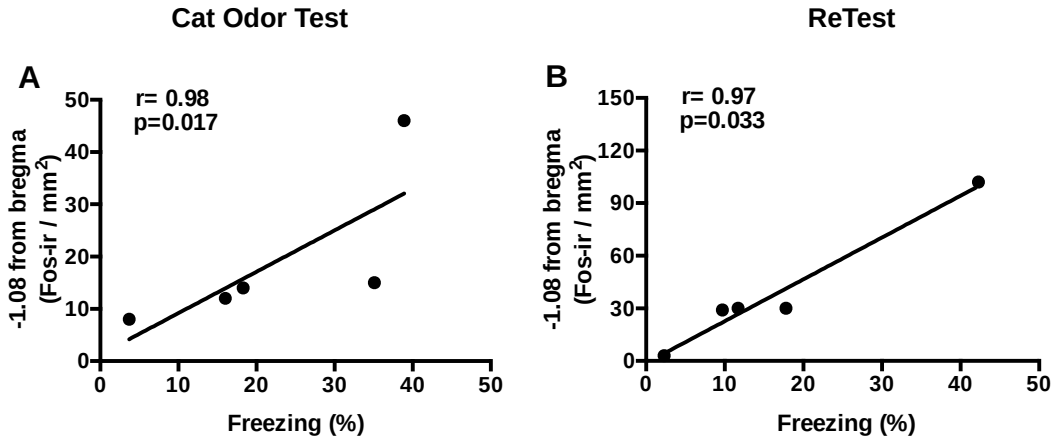


FIGURE Nº18. The neuronal activity of a discrete part of the pIC is highly correlated in response to cat odor. A-B. Correlations between % of freezing over total time and number of Fos-ir neurons in a discrete region of the pIC either in Cat Odor Test and ReTest. The rats were exposed to a control collar (n=5), cat odor for the first time (Cat OdorTest, n=5) or cat odor for the second time (ReTest, n=5). Fos-positive cells were counted at -1.08 from bregma (a discrete region of the pIC). Correlation value and significance is showed in inset. *p<0.05 and Spearman rank order correlation.

6.2.3 Neuronal activity of the key brain structures of the innate fear circuit is correlated with freezing expression.

To confirm that the cat odor is able to activate key brain structures involved in innate fear circuit, we measured Fos activity in the MeApv, a region involved in detection of predator signals and VMHdm, a core integration region for fear to predatory threats (Gross and Canteras, 2012, Silva et al., 2016). As shown in FIGURE Nº19, we also found a significant increase in the number of Fos-ir neurons in the MeApv and in the VMHdm during the cat odor test (FIGURE Nº19A and B; t_{12}=9.274, p<0.0001; t_{12}=4.921, p<0.05; respectively) relative to control collar, as expected from previous works (Blanchard et al., 2005; Silva et al., 2013). During ReTest we also found a significant increase in the number of Fos-ir neurons in the MeApv of the rats exposed to cat odor compared to control collar (FIGURE Nº19A; t_{12}= 4.910, p<0.05), but not in the number of Fos-ir neurons of the VMHdm (FIGURE Nº19B, t_{12}=1.306, p>0.05).

Additionally, we measured Fos activity in LA neurons. This region has not been related to innate fear, but rather has been involved in memory and learned fear (Wallace and Rosen, 2001). We observed no significant changes after cat odor exposure and ReTest (FIGURE Nº19C, p>0.05). Our analysis shows a robust positive correlation in both the MeApv (Spearman r= 0.7 and p= 0.0047, FIGURE Nº19D) and the VMHdm (Spearman r= 0.69 and p= 0.0052, FIGURE Nº19E) between freezing behavior and the number of Fos-ir neurons- LA nucleus did not show a significant correlation: LA (p>0.05, FIGURE Nº19F).

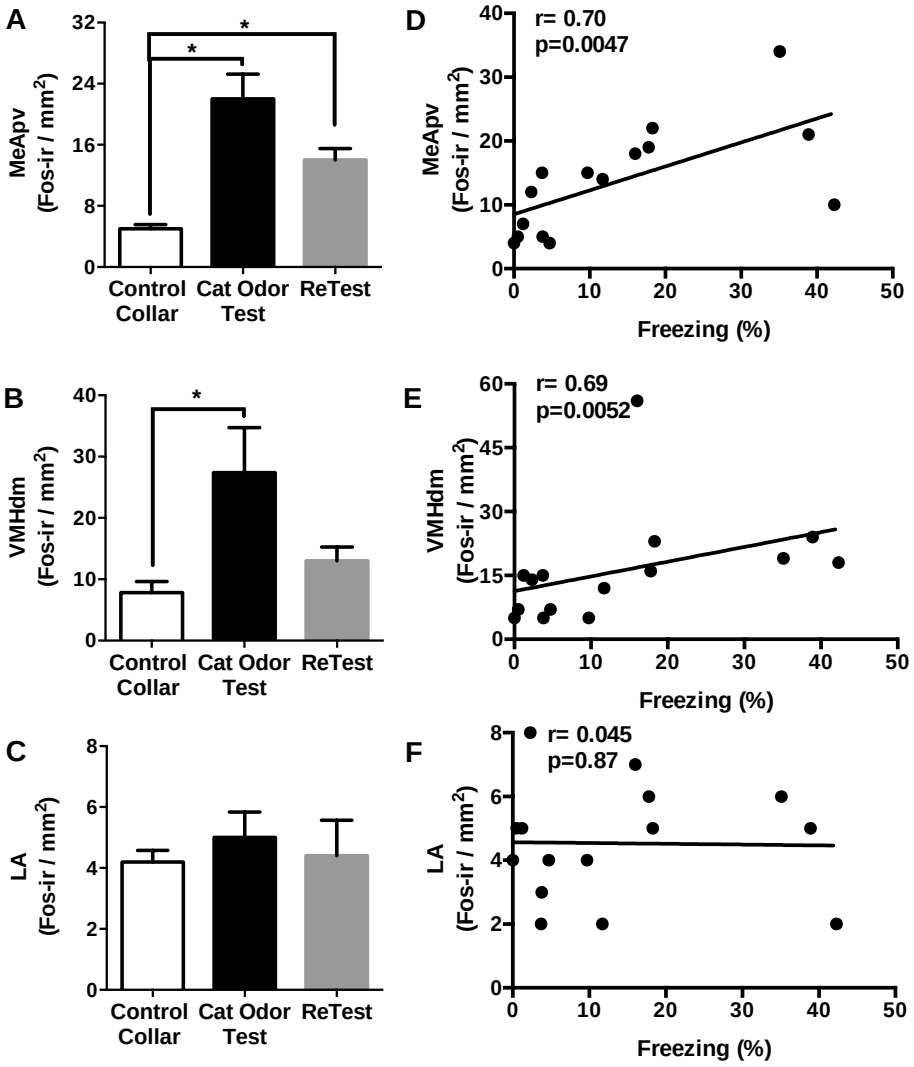


FIGURE Nº19. Effect of cat odor exposure on Fos-ir neurons from the rat in key subcortical nuclei related to innate fear response. A and B. Quantification of Fos-ir in the posteroventral subnucleus of the medial amygdala (MeApv, n=5 for each group), and dorsomedial subnucleus of the ventromedial hypothalamus (VMHdm, n=5 for each group), respectively. C. Quantification of Fos-ir in the lateral amygdala (LA, n=5 for each group) as a negative control region. D and E. Correlations between freezing and Fos-ir neurons in MeApv and VMHdm (correlation value and significance in the inset), respectively. F. No correlation between freezing and Fos-ir neurons in LA.*p<0.05, ****p<0.0001. One-way analysis of variance followed by Tukey test (A-C) and Spearman rank order correlation (D-F).

These data confirmed that the innate fear stimulus increases the neuronal activity in key structures that form part of an innate fear neuronal system in rodents, either on the first or second exposure to cat odor, showing a positive correlation between neuronal activity of each of those regions and freezing behavior.

6.3 Objective 3. Determine the pattern of activity of the pIC using electrophysiological recordings.

6.3.1 Cat odor induced changes in the electrical activity of the pIC.

6.3.1.1 Cat odor Test

This experiment was aimed to explore the activity of single neurons from the pIC during expression of innate fear (FIGURE Nº20A). As shown previously, these rats also evidenced a significant increase in freezing expression in response to cat odor in both time spent and number of episodes (paired t-test, $p<0.01$, FIGURE Nº20B and D). The same observed for risk assessment behavior (paired t-test, $p<0.05$, FIGURE Nº20C and E). The above indicates that innate fear test was successfully experienced.

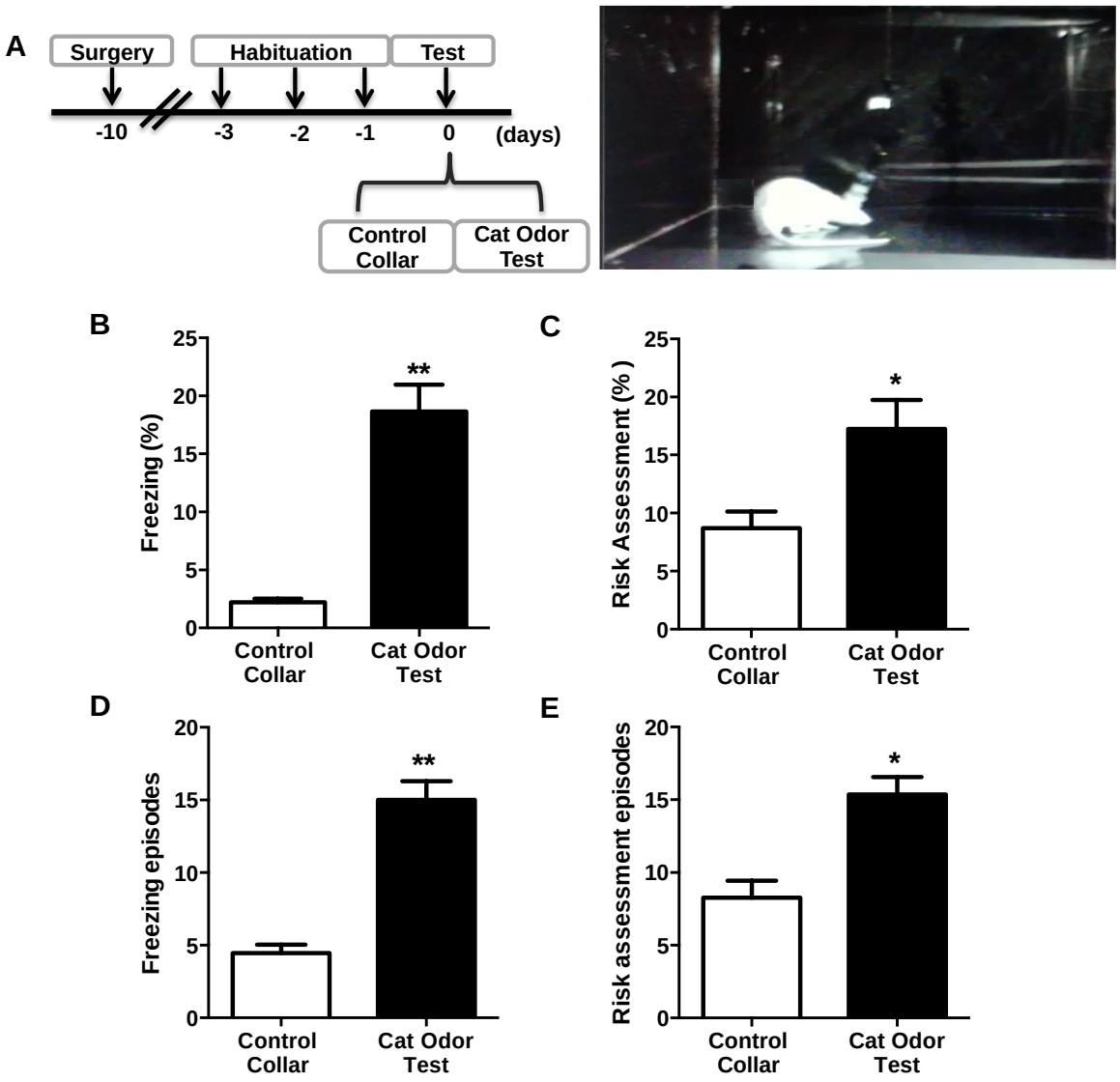


FIGURE Nº20. Cat Odor Test for electrophysiological recordings. A. Experimental design. Rats were exposed 10 minutes for the first time to cat odor immediately after 10 minutes of control collar presentation (familiarized collar). Right, timeline of innate fear protocol. Left, a photograph of rat showing freezing behavior in response to cat odor (left). B-C. % of freezing and risk assessment behaviors in response to control collar (10 min session) and cat odor test (10 min session). D-E Number of episodes of freezing and risk assessment behaviors in response to control collar and cat odor test, respectively Paired t-test, error bars indicate SEM. * $p < 0.05$, **$p<0.01$.

6.3.1.2 Basal pIC recordings

We recorded the activity of the pIC in rats exposed to control collar (first ten minutes) and during exposure to cat odor (last ten minutes of test, FIGURES Nº20 and Nº21). Recordings took place on day 1, during expression of innate fear in rats. A total of 141 well-isolated pIC neurons from 11 rats were included in the analysis to assess their responses to the freezing expression. The exclusion criteria were based on spike waveform, FR and stability of the signal throughout the experiment.

To explore characteristics of the recorded population of single units we analyzed physiological parameter for each single cell and classified these neurons as either putative pyramidal or putative interneurons based on the characteristics of their action potential (AP) waveform. On the resultant waveform, we measure the distribution of waveform peak to trough duration and normalized AP amplitude at 0.46 ms for each cell (SUPPLEMENTARY Nº6A and B). Figure 21B shows the average waveform aligned to peak for all recorded units. Additionally, the peak time in the ISI (inter-spike interval) histogram for each cell was measured. We identified cells with early ISI peaks having many short ISI intervals and cells that had late ISI peaks having many long ISI intervals (SUPPLEMENTARY Nº6C). A K-means clustering algorithm allowed to discriminate two populations of single units. This test revealed a 9% (*n*=12) of putative interneurons and 91% (*n* =129) of putative pyramidal neurons (FIGURE Nº21C).

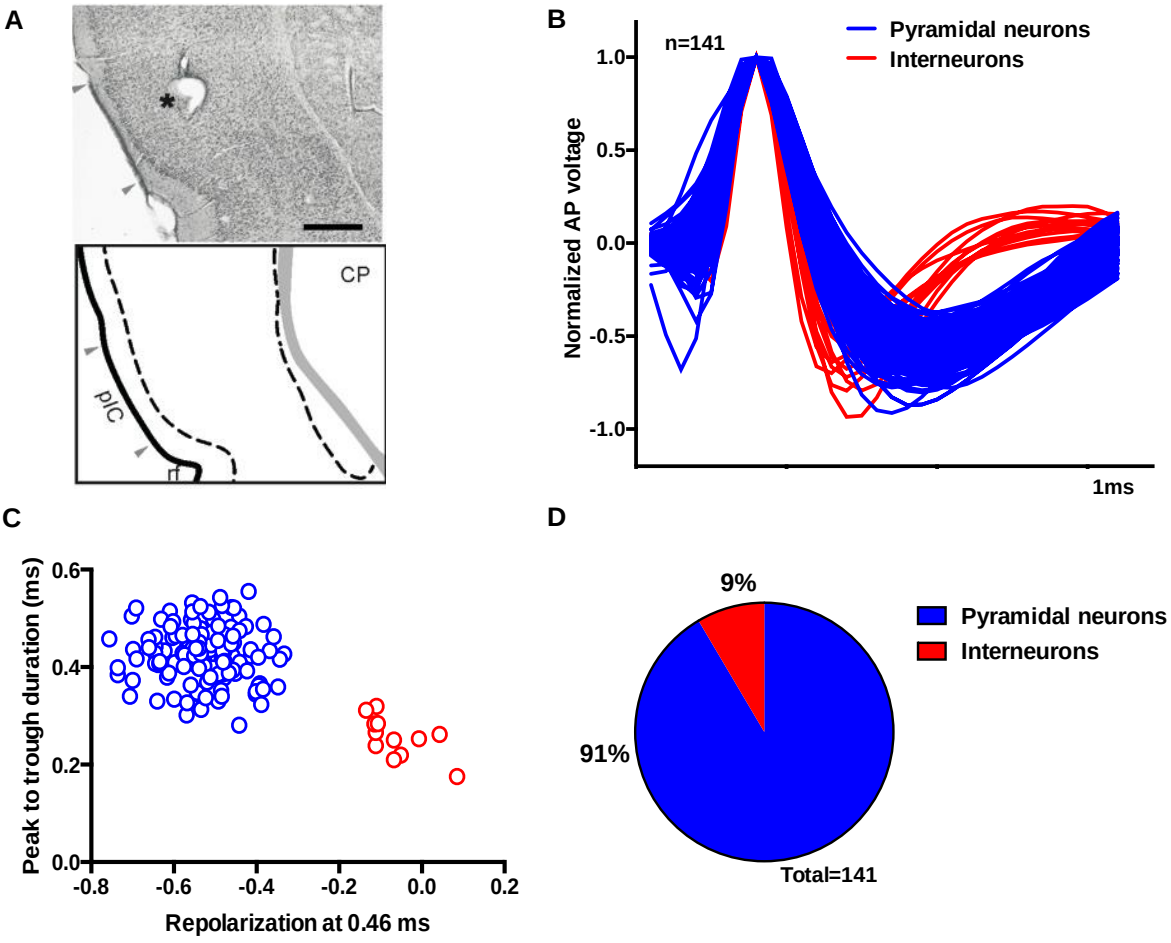


FIGURE Nº21. Extracellular recordings of posterior insular cortex neurons. A. Representative example of recording site. Upper, photomicrograph of a Nissl-stained section; the electrolytic lesion is indicated with an asterisk. Lower, anatomical drawing adapted from Swanson (1998). B. Normalized voltage for all average waveforms of single units recorded. C. Normalized AP amplitude at 0.45 ms (repolarization) vs. waveform peak-to-trough duration (ms), revealing two types of cells. D. Pie chart showing the percentage of units classified as putatitve pyramidal and interneurons. AP, action potential; CP, Caudate Putamen; pIC, posterior insular cortex; rf, rhinal fissure. Scale bar 500 µm.

Since we showed a reduction of freezing behavior by pharmacological manipulations (section 6.1), an incremented activity during cat odor exposure measured by Fos-ir neurons (section 6.2) and a positive correlation between freezing and Fos-ir neurons during cat odor, we reasoned that neurons related to freezing might exist in this brain region.

First at all, we measured global changes of the pIC activity and compared the neuronal activity during Control Collar and Cat Odor Test. A general overview of the variety of responses of pIC neurons is showed in FIGURE Nº22; the heat map depicts the activity of all recorded neurons in decreasing order of average z-score following Cat Odor onset. We observed neurons that increased, decreased or no changed their FR.

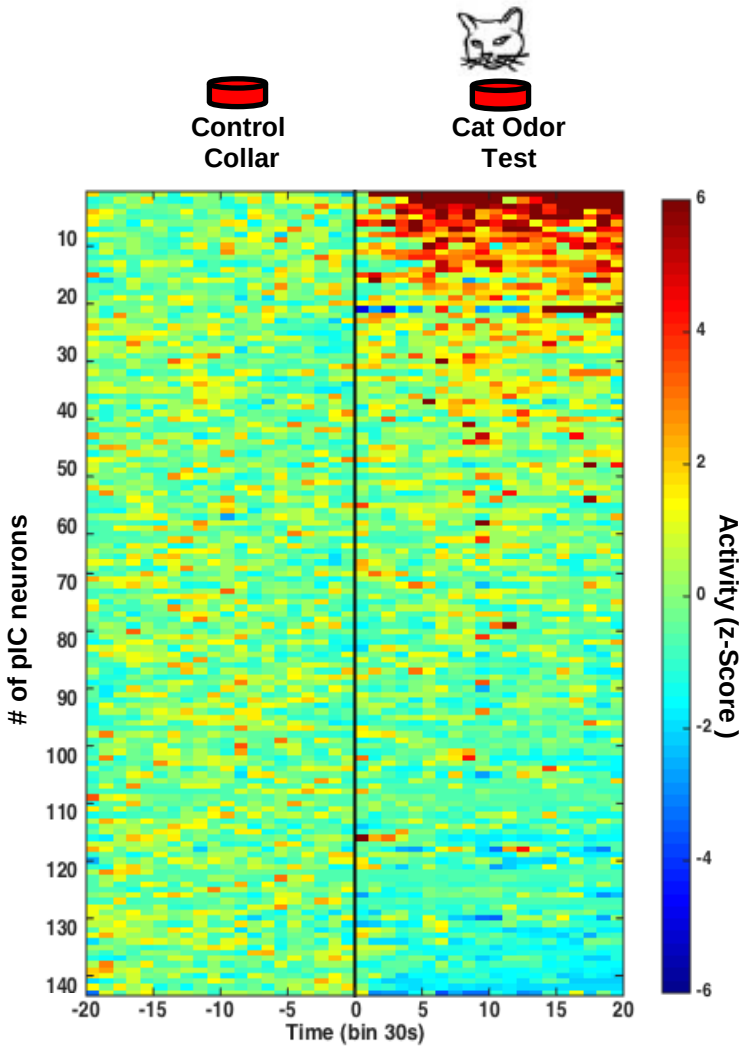


FIGURE Nº22. Neuronal activity of the pIC during Cat Odor Test. Heat plot representing the response of all pIC units to cat odor exposure in descending order of average z-score following cat odor onset. Many cells displayed an abrupt modulation in FR following cat odor presentation. In some cases, changes in the FR were sustained until the end of the recording.

A recently published method (Ardid et al., 2015) was also applied, which classifies cells based on peak to trough times and on repolarization times. The mean FR of those neurons and their normalized AP amplitude at 0.46 ms were evaluated (FIGURE Nº23 and SUPPLEMENTARY Nº7). Interestingly, we found two classes of putative pyramidal neurons: single units that showed higher FR (*n*= 15, a median of 6.1 Hz with a range of 4.2 to 18.9 Hz) and single units that showed lower FR (*n*=114, a median of 0.62 Hz with a range of 0.1 to 3.7 Hz). Putative interneurons showed median rank estimated of 1.26 Hz, with a range of 0.1 to 13.6 Hz, which is in agreement with previously reported data for the pIC (Hanamori, 2005). TABLE Nº2, see appendix, shows the percentage and number of cell that exhibiting statistically significant changes in FR in response to cat odor, considering this new classification.

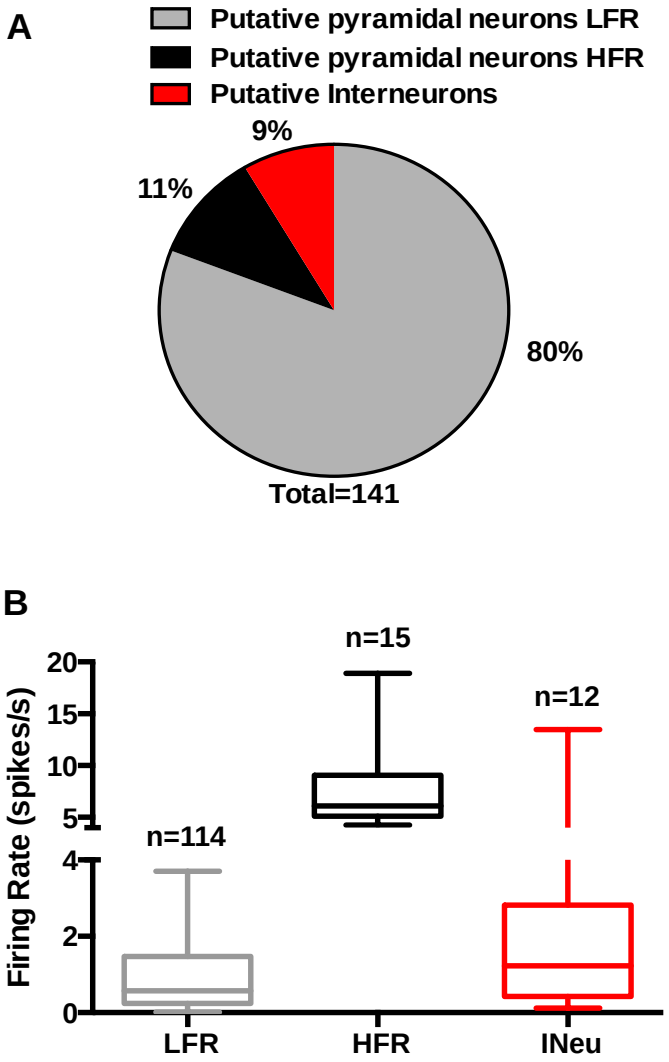


FIGURE Nº23. Two classes of putative pyramidal cell in pIC of the rat. Normalized AP amplitude at 0.46 ms v/s firing rate, revealed three electrophysiological cell classes. K indicated 4.2 Hz. A Showing proportion of cell classes. B Showing median FR for each electrophysiological class. LFR were putative pyramidal cell showing a low firing rate; HFR were putative pyramidal cell showing a high firing rate and INeu as interneurons. Note the most of neurons belong to LFR class.

6.3.2 Differential pattern of pIC activity during expression of defensive behaviors

Freezing behavior

To determine significant changes of the FR for each single unit during innate fear behavior, we computed the expected activity difference employing the permutation test (see methods, section 5.5.1.2). The average freezing duration per episode was 7± 0.946 s in response to cat odor, while 3± 0,453 s in response to cat collar. Thus, we selected freezing episodes ≥ 3s for running the permutation test.

We found that ~35% (49/141, FIGURE Nº24A) of neurons changed their FR in response to cat odor, aligned to the onset of innate freezing expression relative to IBI. We found a 23% (32/141) of neurons with higher FR than IBI (median: 1.83 [interquartile range: 0.68-3.75] spikes/s vs median: 0.96 [0.38-1.79] spikes/s, FIGURE Nº24B), and a 12% (17/141) of neurons with a significant decrease of their FR relative to IBI (median: 0.35 [interquartile range: 0.1-0.77] spikes/s vs median: 0.77 [interquartile range: 0.45-1.52] spikes/s, FIGURE Nº24C).

We identified interneurons (INeu n=2, median: 9.93 [interquartile range: 3.5-16.35] vs 5.22 [interquartile range: 1.18-8.62] spikes/s; FIGURE Nº24D) and two classes of pyramidal neurons (LFR; n=26, median: 0.97 [interquartile range: 0.61-2.23] vs 0.59 [interquartile range: 0.36-1.6] spikes/s and HFR; n=4, median: 7.5 [interquartile range: 5.16- 9.27] vs 2.77 [interquartile range: 0.74-6.9] spikes/s; FIGURE Nº24D) that increased their FR aligned to onset of freezing behavior relative to IBI.

We also found interneurons (INeu n=2, median: 0.41 [interquartile range: 0.37-0.45] vs 0.75 [interquartile range: 0.57-0.93] spikes/s; FIGURE Nº24E) and two classes of pyramidal neurons (LFR; n=14, median: 0.27 [interquartile range: 0.09-0.76] vs 0.70 [interquartile range: 0.38-1.52] spikes/s and HFR; n=1, median: 11.38 vs 16.98 spikes/s; FIGURE Nº24E) that decreased their FR during freezing relative to IBI.

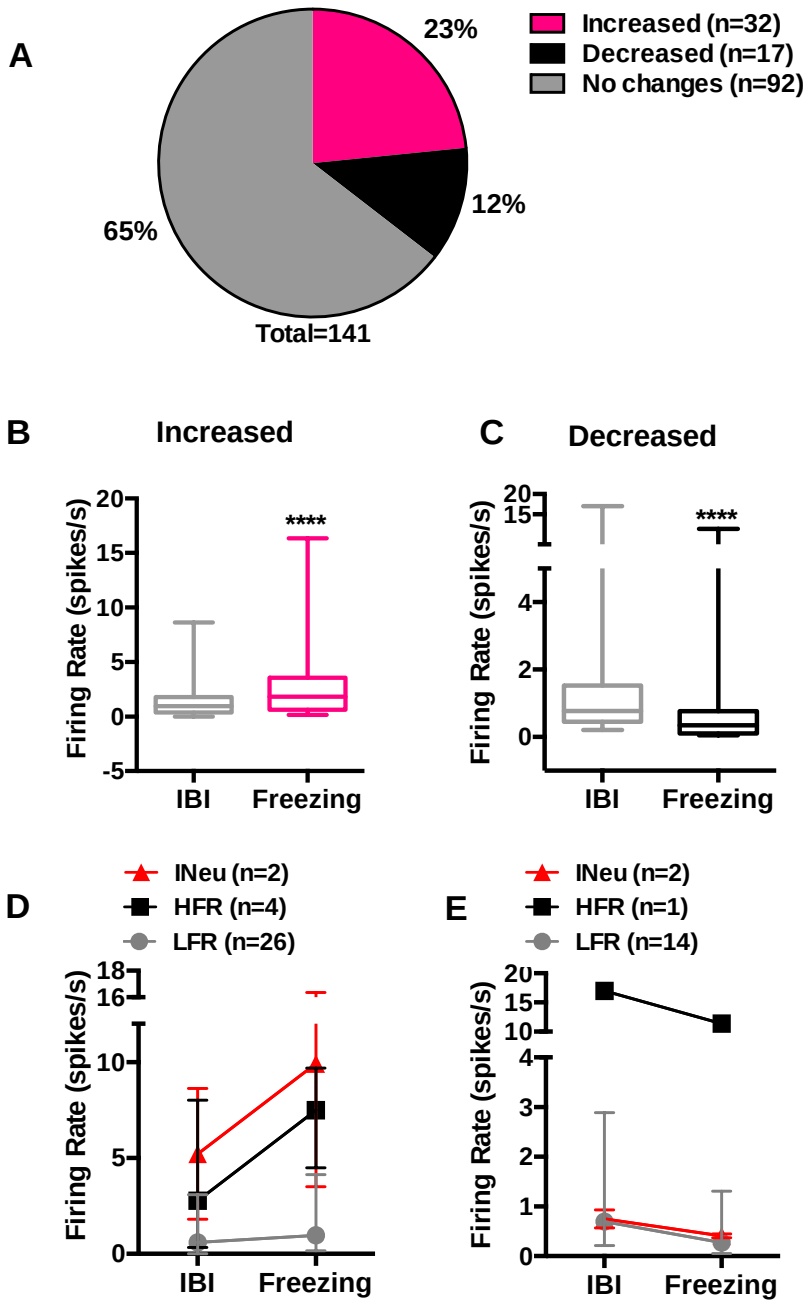


FIGURE Nº24. Changes in the FR during innate fear expression in response to cat odor. A Pie chart illustrating the percentage of pIC neurons shows an increase, decrease and no change in their activity during freezing expression. B. Single units that increased their FR relative to IBI (inter-behavior interval). C Single units that decreased their FR relative to IBI. D-E Showing pIC neurons that increased and decreased for each cell class. Data are represented as box and whiskers, showing the median (middle line) and the 25th and 75th percentile in the box, and the lowest and highest values (whiskers); ****p < 0.0001, Wilcoxon signed rank test.

FIGURE Nº25A and B illustrated representative raster plots of single-units recorded that exhibited significantly changes in their FR during the onset of freezing expression. As well as, we found single units that did not modify their FR during the onset of freezing (FIGURE Nº25C).

The neuronal activity shown as average z-score for 35% (49/141) of units that showed significant changes relative to IBI is indicated in FIGURE Nº25D. Most of pIC unit responses tended to increase or decrease transiently during innate freezing expression induced by cat odor.

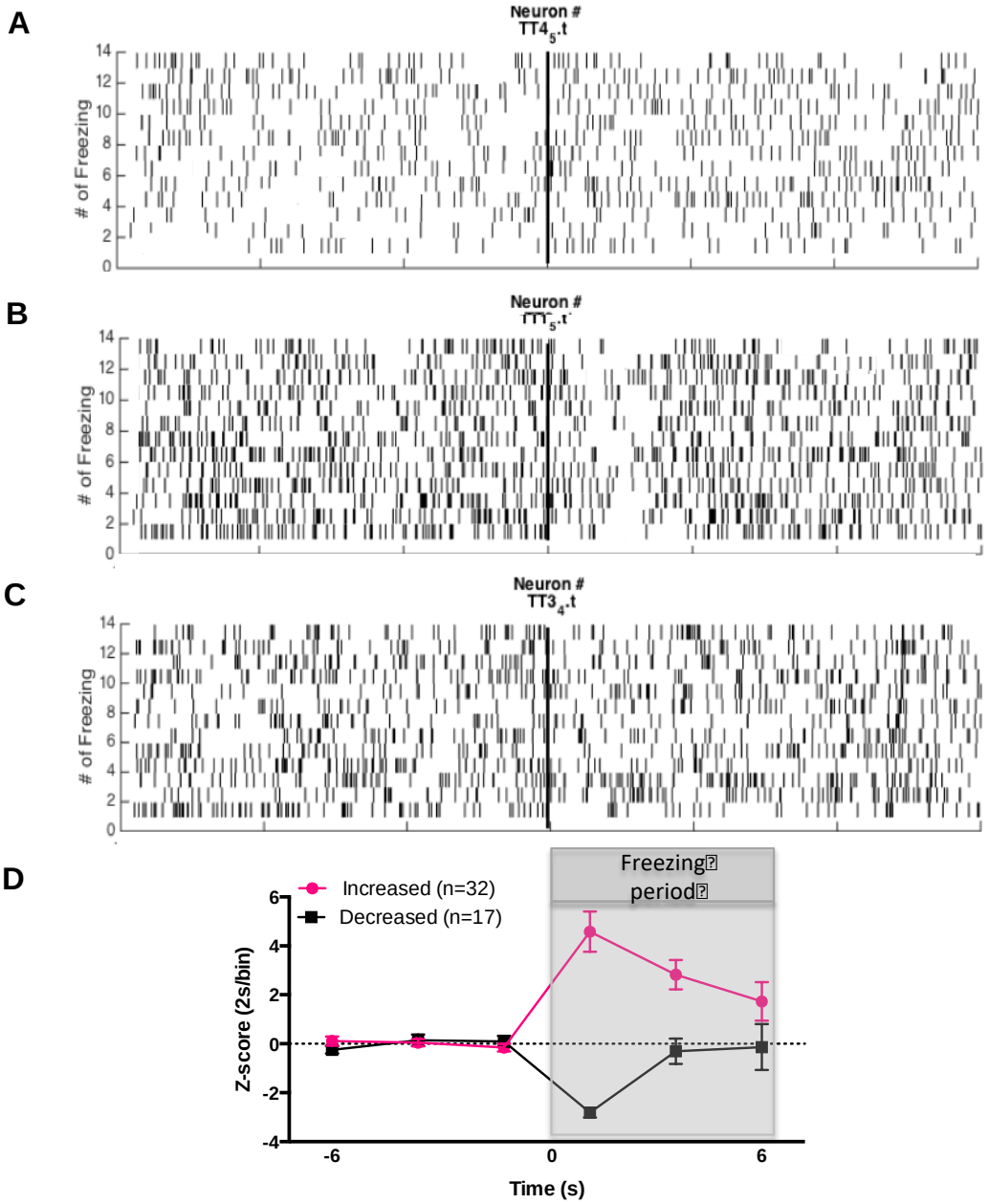


FIGURE Nº25. Responsiveness of pIC units during innate fear expression. A-C. Representative spike trains of three neurons that incremented (A), decreased (B) or no changed (C) their FR during freezing period (6s). Black vertical line indicates the beginning of freezing episodes D. Average FR (z-score, 2s/bin) of pIC units showing an increase or decrease during freezing period. Data are represented as mean +/-SE.

Risk Assessment behavior

To determine significant changes of the FR for each single unit during innate anxiety behavior, we computed the expected activity difference employing the permutation test (see methods, section 5.5.1.2). The average risk assessment duration per episode was 7± 0.803 s in response to cat odor, while 7± 0.748 s in response to cat collar. Thus, we selected risk assessment episodes ≥ 3s for running the permutation test, to compare the neuronal activity, in a similar time frame, associated in both freezing and risk assessment behaviors.

We found that ~38% (54/141, FIGURE Nº26A) of neurons changed their FR in response to cat odor, aligned to the onset of innate risk assessment expression relative to IBI. We found a 26% (36/141) of neurons with higher FR than IBI (median: 2.61 [interquartile range: 2.79-5.45] spikes/s vs median: 1.94 [1.88-3.73] spikes/s, FIGURE Nº26B), and a 13% (18/141) of neurons with a significant decrease of their FR relative to IBI (median: 0.09 [interquartile range: 0.03-0.53] spikes/s vs median: 0.50 [interquartile range: 0.25–1.27] spikes/s, FIGURE Nº26C).

We identified interneurons (INeu n=2, median: 11.68 [interquartile range: 7.00-16.35] vs 4.48 [interquartile range: 0.34-8.62] spikes/s; FIGURE Nº26D) and two classes of pyramidal neurons (LFR; n=26, median: 2.16 [interquartile range: 0.08-5.47] vs 1.20 [interquartile range: 0.03-3.73] spikes/s and HFR; n=8, median: 8.78 [interquartile range: 5.27-3.18] vs 6.54 [interquartile range: 3.58-9.65] spikes/s;

FIGURE Nº26D) that increased their FR aligned to onset of risk assessment behavior relative to IBI.

We also found interneurons (INeu n=3, median: 1.80 [interquartile range: 0.54-2.03] vs 2.99 [interquartile range: 1.01-3.71] spikes/s; FIGURE Nº26E) and pyramidal neurons (Low FR; n=15, median: 0.08 [interquartile range: 0.01-2.03] vs 0.45 [interquartile range: 0.11-2.99] spikes/s; FIGURE Nº26E) that decreased their FR during risk assessment behavior relative to IBI.

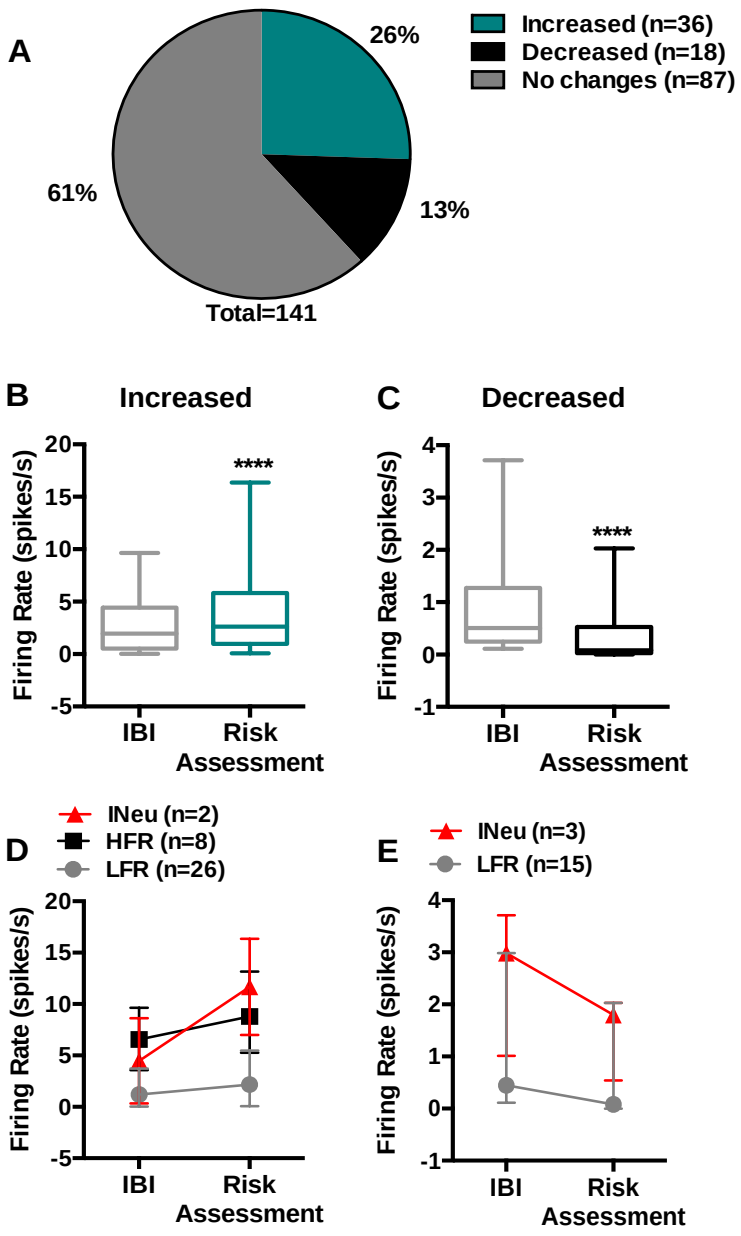


FIGURE Nº26. Changes in the FR during innate anxiety expression in response to cat odor. A Pie chart illustrating the percentage of pIC neurons shows an increase, decrease and no change in their activity during risk assessment expression. B. Single units that increased their FR relative to IBI (inter-behavior interval). C Single units that decreased their FR relative to IBI. D-E Showing pIC neurons that increased and decreased for each cell class. Data are represented as box and whiskers, showing the median (middle line) and the 25th and 75th percentile in the box, and the lowest and highest values (whiskers); **p < 0.01, Wilcoxon signed rank test.

FIGURE Nº27A and B illustrated representative raster plots of single-units recorded that exhibited significantly changes in their FR during the onset of freezing expression. As well as, we found single units that did not modify their FR during the onset of freezing (FIGURE Nº27C). The neuronal activity shown as average z-score for 37% (54/141) of units that showed significant changes relative to IBI is indicated in FIGURE Nº27D. Most of pIC unit responses tended to increase or decrease in a sustained way during innate risk assessment expression induced by cat odor.

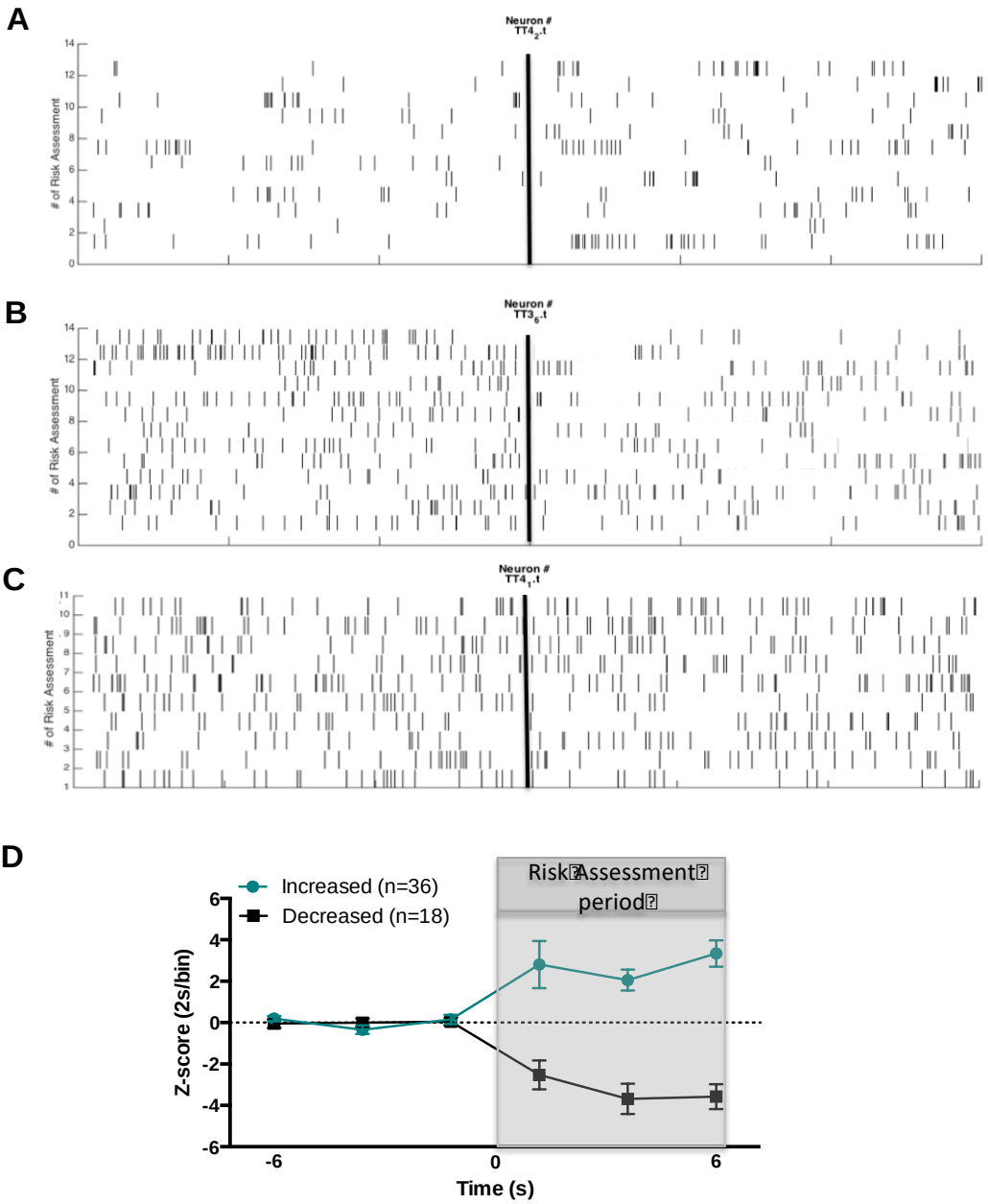


FIGURE Nº27. Responsiveness of pIC units during innate anxiety expression. A-C. Representative spike trains of three neurons that incremented (A), decreased (B) or no changed (C) their FR during risk assessment period (6s). Black vertical line indicates the beginning of risk assessment behavior. D. Average FR (z-score, 2s/bin) of pIC units showing an increase or decrease during risk assessment period. Data are represented as mean +/-SE.

TABLE Nº3 showed the percentage of cells excited or inhibited for all neurons recorded during each defensive behavior. We found that 35% (49/141) of neurons changed their firing rate associated to the innate fear expression and other 38% (54/141) during innate anxiety-like behavior induced by cat odor, with only a 18% (25/141) of total units responsive specifically during freezing episodes while 21% (30/141) of total units responsive specifically during risk assessment episodes. Interestingly, we detected a population of neurons that modify significantly their FR during freezing and risk assessment.

Complementary, we showed in TABLES 4-6 the percentage of cells excited or inhibited during freezing, risk assessment or both for each cell class. For both types the proportion of responsive cell during defensive behaviors was similar showing around a 60% for each cell class. Note that proportion of HFR neurons that change their FR during risk assessment expression is higher than responsive HFR neurons during freezing behavior. We found a subset of HFR pyramidal neurons that had an excitatory response during risk assessment episodes (8/15), which only 4 units of these were exclusively responsive during risk assessment and the 4 remaining cells were responsive during freezing and risk assessment behaviors.

Interestingly, we did not find HFR neurons with excitatory response during freezing behavior and we only found one neuron that inhibits its activity from the onset of this behavior.

7.DISCUSSION

7.1 Effect of pIC inactivation on behavioral pattern induced by cat odor.

Several lines of evidence have reported a contribution of the IC in fear or anxiety in humans (Phelps et al., 2001, 2004; Morris and Dolan, 2004; Alvarez et al., 2008; Paulus and Stein, 2010) and rats (Alves et al., 2013; Reed et al., 2013; Li et al., 2014; Casanova et al., 2016), but the relevance of the pIC as a site for the innate fear expression have not been explored. To date, there is evidence that the amygdala is not required for the conscious experience of fear (Feinstein et al., 2013; Khalsa et al., 2016); therefore, a neocortical region, such as the IC, may be a candidate as a hub that links sensory and physiological aspects of the fear experience to contribute to the perception of fear.

In the present work, we demonstrate for the first time that IC plays an important role in the expression of innate as well as learned defensive responses to predator odor. We found that the Muscimol-induced inactivation of the pIC reduced the expression of freezing reaction to a single or repeated exposure to cat odor (FIGURE Nº12 and 15). We also found that the pIC inactivation with Muscimol impaired fear

conditioning to the context in which rats were previously exposed to cat odor (FIGURE Nº15). Furthermore, long-term pIC inactivation (NSTX) resulted in a prolonged and robust reduction in freezing response in subsequent re-exposures to cat odor (FIGURE Nº14B). By contrast, the reduction in risk assessment lasted no longer than 24 hours after Neosaxitoxin injection into pIC (FIGURE Nº 14C).

A growing body of evidence suggests that the interoceptive insular cortex plays an important role in learned fear responses. We have recently reported that pretraining inactivation of the pIC or the intra-pIC blockade of protein synthesis immediately after training impaired the consolidation of auditory fear conditioning (Casanova et al., 2016). Here, we show that the pIC inactivation reduced the expression of innate fear and impaired the contextual memory to predator odor, similar to the well-known effects of interfering with specific subcortical regions responsible for innate defensive behavior.

Exposure to predator odor is a natural stressor for rodents that is known to activate the animal's defensive system (Dielenberg et al., 2001a, b; Apfelbach, et al., 2005; Takahashi, et al., 2005; Papes et al., 2010; Gross and Canteras, 2012). For example, acute exposure of rats to cat odor induced a substantial increase in the neuronal activity in both MeApv and VMHdm, and defensive responses including freezing and risk assessment (Blanchard et al, 2005; Pérez-Gómez et al., 2015). Lesions of the MeA reduced freezing either to predator odor (Li et al., 2004; Müller and Fendt, 2006) or to a live cat (Martinez et al., 2011), and the optogenetic stimulation of VMHdm generated autonomic and behavioral responses that resemble the animals' natural defensive behaviors (Wang et al., 2015). Interestingly, the IC is heavily connected to key

structures of the defensive system, including MeA, VMHdm, prefrontal cortex, and midbrain sites such as the periaqueductal gray (Canteras et al., 1994; Canteras et al., 1995, Shi and Cassell, 1998), but it is not known whether IC interacts with these regions for modulating defensive behaviors.

Functional studies indicate that IC is important for representing the visceral state of the body, which may influence decision-making, motivation, and cognitive and emotional processes (Craig, 2004; Garfinkel and Critchley, 2016; Damasio and Carvalho, 2013). It is well known that numerous interoceptive changes take place during fear response. For instance, increments in heart rate, blood pressure (Le Doux, 1988; Dielenberg and McGregor, 2001) and plasma glucocorticoid levels (Figueiredo et al., 2003) have been observed in rats exposed to predator odor. These autonomic and endocrine changes (i.e. fear-related bodily state) may be represented in the interoceptive network, initially in the nucleus tractus solitarius (Claps and Torrealba, 1988; Torrealba and Claps, 1988), and after within insular cortex. Studies in rats (Contreras et al., 2007; Contreras et al., 2012) and humans (Craig, 2004; Critchley and Harrison, 2013) have suggested that pIC map viscerosensory state, while anterior insula, which is reciprocally interconnected with the pIC, is associated with the representation of visceral sensations and interoceptive memory. We hypothesized that the sensory representation of fear-related bodily state in pIC and subsequently into anterior insula may modulate defensive responses to predator odor.

In support of this idea, previous work has shown that fear and anxiety responses may be modulated by afferent visceral signals. Rats with subdiaphragmatic vagal

deafferentation showed reduced anxiety levels and an attenuation of conditioned fear extinction (Klarer et al., 2014). Moreover, imaging studies have shown that changes in the cardiorespiratory state are represented in the insula (Hassanpour et al., 2017), fear processing is modulated by cardiac activity (Garfinkel et al., 2014), and human fear-related disorders such as anxiety are associated to an altered interoceptive processing in the insular cortex (Paulus and Stein, 2006, Simmons et al., 2011).

The finding that the Muscimol-induced inactivation of the pIC reduced innate fear expression and impaired the contextual memory to predator odor appears compatible with the modulatory role of the IC on the defensive responses, and with previous studies in rats showing that the inactivation of the anterior IC immediately after training attenuated the behavioral and cardiovascular responses to the training context, suggesting the involvement of the IC in the consolidation of contextual fear memory (Alves et al., 2013). Moreover, we previously reported that the inactivation of the pIC increased the latency to express gastric malaise and disrupted drug craving (Contreras et al., 2007), and that the anterior insula is involved in context/drug effect association (Contreras et al., 2012) as well as in consolidation of auditory fear memory (Casanova et al., 2016).

Although Muscimol-induced inactivation of the pIC prior to cat odor exposure resulted in a robust reduction of freezing behavior, no significant effect was observed on risk assessment. However, injection of the NSTX into pIC immediately after the first cat odor exposure, in addition to cause a long-lasting reduction in freezing expression

in subsequent exposures, it reduced risk assessment behavior for 24 hours. These findings suggest that pIC influences innate defensive reactions mainly in situations where the animal is dealing with high predation risks, but not when the threatening stimuli becomes ambiguous and unlocalized, and it is necessary to assess predation risk in order to deploy appropriate defensive behaviors. We also suggest a possible effect of the long-term SS inactivation on fear behavior, denoted as a reduction in freezing behavior in subsequent re-exposures to cat odor. We reasoned that connectivity between pIC and SS (Mufson and Mesulam, 1984; Shi and Cassell, 1998) could reduce the freezing expression around four days after microinjection into the pIC.

Muscimol injected into pIC prior to cat odor exposure impaired contextual fear conditioning. Notably, we observed a reduction in both freezing and risk assessment when rats were re-exposed to the cat odor-paired context 24 hours after Muscimol injection. As discussed above, IC has been implicated in consolidation of contextual and auditory-cued fear conditioning to footshock (Alves et al., 2013; Casanova et al., 2016). Together, these data indicate that the IC may be required for encoding fear learning of environmental cues that were previously associated with innate fear stimuli (such as predators) or with physically harmful or aversive stimuli (such as footshocks).

The present results point to a necessary role for the pIC in the expression of innate and learned fear. Is possible that pIC contains early in life an innate association between cat odor and a constellation of bodily responses that are processed as fear.

7.2 Cat odor induces neuronal activity in the Insular Cortex

The neuronal activity of the pIC during defensive behaviors had not been studied. The present study was undertaken to address this subject by neuronal activity mapping using immunohistochemistry to measure Fos protein levels (an early gene marker) and by extracellular single-unit recordings from the pIC during the time course of defensive behaviors in rats exposed to cat odor.

7.2.1 Immunohistochemistry measures.

Using an immunohistochemical approach, we confirmed that cat odor inducer increases Fos-ir levels in the IC, in both pIC and RAIC (FIGURE Nº16B) as well as in key brain structures that form part of an innate fear neuronal system in rodents (FIGURE Nº19), which indicates that our stimulus used has innate threat-like properties and it was enough to trigger predator fear system.

Studies in rats have shown that the pIC is reciprocally connected with the RAIC, the most rostral part of the anterior domain of the IC in the rat, through an intermediate region of insula, giving RAIC a higher hierarchical order in interoceptive processing (Shi and Cassell, 1998); therefore, a bidirectional flow of bodily-related information between the pIC to the RAIC seems plausible (Cauda et al., 2012; Shi and Cassell, 1998) necessary for the emotional experience (Craig, 2003) and learning (Alves et al., 2013; Contreras et al., 2012).

We also found that freezing behavior either, innate or learned (FIGURE Nº18), is correlated with a discrete area of the pIC, but not between risk assessment and pIC neuronal activity (SUPPLEMENTARY Nº5), suggesting it may be relevant in processing interoceptive components of fear.

Rodent studies have reported that interoceptive information of gastric mechanoreceptors, respiratory chemoreceptors and cardiovascular baroreceptors reaches pIC levels at 0.0 to -1.78 from bregma, through VPLpc the interoceptive thalamic subnucleus (Cechetto and Saper, 1987). The level -1.08 from bregma receives afferents mainly from VPLpc, the interoceptive thalamic subnucleus and local intracortical afferents: from granular, disgranular and agranular fields (Cechetto, 1990; Madrid, 2004). Moreover, the RAIC has strong bidirectional connections with the amygdala (Shi and Cassell, 1998), prefrontal cortices and hippocampus, which could explain the correlation between freezing behavior and of the global activity of the anterior insula as well as the global activity of the pIC with after second exposure (FIGURE Nº17).

Consistent with previous reports, a single cat odor exposure elicited a robust increase in Fos-ir neurons in both MeApv and VMHdm (Gross and Canteras, 2012), and also Fos-ir within these regions was correlated with freezing either in presence or not of the threatening stimulus. A second cat odor exposure elicited a similar pattern although with less activation in the VMHdm, as shown before (Staples et al., 2005). The reduced activation of the VMHdm during the second exposure could be due to a change in stimulus processing that occurs when the stimulus becomes familiar.

The relationship between MeApv activity as well as VMHdm activity and those discrete parts of RAIC and pIC suggest a possible contribution of the IC in the predator fear circuit. The no relation between risk assessment behavior and Fos-ir of the IC is supported by data obtained in this thesis. Structures such as prefrontal cortices, lateral septum, hippocampus can participate in this anxiety-related behavior (Gray and McNaughton, 2000).

Here, we propose the modulation of defensive behaviors, observed in sections 6.1 and 6.2 by IC activity in rats based on its connections. A strong visceral input from the thalamus along with key structures mediating innate fear responses, such as the MeA (Canteras et al., 1995) and VMH (Canteras et al., 1994), as well as structures involved in planning and memory as prefrontal cortices, hippocampus, lateral amygdala, among others (Allen et al., 1991; Shi and Cassell, 1998). Moreover, the interoceptive flow of information from the pIC towards anterior subdivision would involve the integration of emotional and cognitive aspects of information in the anterior IC (Cechetto and Saper, 1987). This entire connectivity pattern posits the IC as a plausible site mediating autonomic and behavioral responses to innate fear inducer.

In this sense, the pIC activation in response to cat odor, would induce a corresponding activation of the RAIC, which together with the activation of other cortical structures would arise the perception or feeling of fear.

7.2.2 Electrophysiological recordings.

We performed extracellular single-units' electrophysiological recordings in behaving rats, to measure the activity of the pIC neurons during innate defensive behaviors in response to cat odor (FIGURES Nº20 and 21).

This present work shows single units with significant changes in the FR during both innate fear and anxiety expressions (i.e. freezing and risk assessment behaviors) compared to non-behavior periods. We found differences in how pIC encodes the interoceptive information associated to fear and anxiety. One population of neurons was responsive during freezing expression showing a transient change in the FR from the onset of freezing (FIGURES Nº24 and 25), while another population was responsive during risk assessment behavior showing a sustained activity of the FR during this behavior (FIGURES Nº26 and 27). Furthermore, we found a third population in which the same unit was responsive during freezing and risk assessment behaviors (TABLES Nº2 to 5).

Multiple behavioral processes are orchestrated to display complex behavioral patterns. Electrophysiological studies in rats have shown the participation of IC in the olfactory perception related to taste stimulus (Maier et al., 2013), in absence of the taste stimuli (Samuelsen and Fontanini, 2017) as well as in responses to pleasant and unpleasant odor (Rolls et al., 2003). As discussed before, the posterior insula is considered a site of visceral and cardiovascular control. It has been demonstrated that the neurons in the posterior insula are responsive to baroreceptor and chemoreceptor stimulation between 0.0 to -1.78 from bregma, inducing changes in the BP and, in reverse, changes in BP induce an excitatory or inhibitory response in neuronal activity of the pIC (Ruggiero, et al., 1987; Yasui, et al., 1991). Therefore, a specific interoceptive

pattern that underlie to bodily manifestations during fear and anxiety (HR, BP, temperature, pH, etc.) could be represented by changes in the pIC activity pattern during behavioral expression in response to cat odor exposure.

Here, we identified subsets of units that significantly modified their FR exclusively during freezing, other units exclusively during risk assessment and a third subset that was responsive for both behaviors.

It is well known that pyramidal neurons and the high diversity of interneurons establish cortical microcircuits which integrate, synchronize and coordinate the balance of excitation and inhibition of subordinate as well as its own microcircuits. Recently, it has been proposed criteria for classifying pyramidal cells onto variety of classes, given the idea of that microcircuits are composed of multiple cell classes that likely serve unique circuit operations (Ardid et al., 2015). Here, we explored the above and through a supplementary analysis, putative interneurons and two functional classes of putative pyramidal cells were found. The mostly of units recorded into the pIC correspond to pyramidal neurons with low FR (LFR, 81%) as that previously described (Hanamori, 2005; Casanova, 2016), whereas the proportion of pyramidal neurons with high FR (HFR) correspond to 11%.

Interestingly, we found a subset of putative HFR pyramidal neurons that only had an excitatory response exclusively during risk assessment episodes (4/15), while other 4 units incremented their activity during freezing and risk assessment simultaneously but none of these neurons modified their FR uniquely during freezing

behavior. The above suggests that pIC could has a specific population with high frequency code for anxiety information bodily related.

A low proportion of putative interneurons (INeu, 9%) relative to putative pyramidal neurons were found, showing heterogeneity in the FR similar to that reported for the orbitofrontal cortex (Quirk et al., 2009), a region considered to be an extension of the anterior insula (Jasmin et al., 2004).

Fear and anxiety can be understood as brain states that are caused by external or internal stimuli and that trigger a specific set of measurable behavioral and physiological reactions. Neurons that significantly changed during innate fear expression, showed an acute response in their FR pattern. In contrast, pIC neurons that modified their FR during risk assessment exhibited a persistent change in the FR during the time course of this behavior. We think that differences observed in the pIC activity in response to cat odor during innate fear and anxiety behaviors are regarding to how pIC codes bodily information associated to fear and anxiety. These processing can be consequence of the pIC organization and their connections with other structures implicated in behavioral planning and execution, spatial navigation as well as recognizing and processing of the threatening stimulus. Structures such as prefrontal cortex, hippocampus and subcortical nuclei involved in innate defensive system, for example.

Rodent and human studies have reported that coupling of neuronal oscillations in the gamma and theta bands are important in the emotional processing between subcotical and cortical structures (Stujenske et al., 2014; Luo et al., 2013). Therefore, we reasoned would be relevant execute further analysis of the oscillations focused on

the study of coupling of gamma and theta bands from pIC and subcortical fear-related structures, during fear and anxiety bodily states in response to cat odor.

Unpredictable and predictable threats induce similar signs of defense but elicit distinct behavioral and neuronal responses. Risk assessment as previously described, is a behavior that characterizes an anxiogenic state oriented to explore the environment for evaluating the novel stimuli which have both threatening and curiosity-inducing aspects. This ambiguity elicits the approach/avoidance conflict (Gray and McNaughton, 2000), but not on approach or avoidance as such but on the conflict between them. With these the same stimulus tends to elicit both approach (curiosity driven) and avoidance (potential threat driven). In contrast, freezing is a behavior that represents a fearful state, elicited by recognizing the stimulus as completely threatening, indicating the intensity of stimulus, which is elicited by an intermediary defensive distance the animal cannot escape, thus the preparatory drive is towards freeze (Blanchard et al., 1990).

Although a detailed correspondence between the human and rat insula is still lacking (Craig 2009), it is worth mentioning evidence linking the activity of the insula with emotion perception (Damasio et al. 2000). Evidence from fMRI shows that the predictable threat cue evoked transient activity in IC and amygdala as well as other frontal and parietal structures, while unpredictable threat produced sustained activity in anterior insula bed nucleus of the stria terminalis (BNST) complex and other frontal structures (Alvarez et al., 2011; Brinkmann et al., 2017).

Extracellular electrophysiological recording from our lab, in conditioned fear to an auditory stimulus (Casanova, 2016), showed a persistent response during time course of freezing behavior; in contrast, here, we observed a transitory change in the FR during the onset of innate freezing behavior. It is probably that this difference might represent the persistence in fear memory.

Our results obtained from pIC inactivation's and electrophysiological recordings, allow suggest that the acute and transitory change of the neuronal activity from the pIC associated to freezing behavior in response to cat odor, could represent the neuronal coding of interoceptive signals relevant for the perception of imminent danger. However, considering the sustained change in the FR, observed in our single-units' recordings, during risk assessment behavior and our results obtained by acute inactivation with Muscimol in a single exposure to cat odor, we suggest that pIC activity would not sufficient to decrease the occurrence of this behavior, but is possible with long term inactivation, which could be relevant to promote fear memories.

In rats, the anterior part of the insula, RAIC, has been attributed as integration zone due to it high brain connectivity and it has reported that this region is bilaterally connected with olfactory cortex (Shipley and Geinisman, 1984) and MeA nucleus (Canteras et al., 1995). We did not discard that neurons that responded during freezing may participate in liking odorant with interoceptive signals. Optogenetics approaches are necessary to uncover whether integration between emotion-related perception and behavioral response take place in the pIC, as described for studies of innate defensive system (Deng et al., 2016; Falkner et al., 2016; Kunwar et al., 2015; Wang et al., 2015).

A flow of information from the posterior to anterior insula has been proposed (Cauda et al., 2012), allowing the integration between homeostatic and more complex cognitive and emotional information in the anterior insula. This integration underlies the enhancing of the physiological awareness of salient stimuli in order to organize behavior (Menon and Uddin, 2010). Accordingly, the continuous monitoring of the physiological condition of the body, exerted by the interoceptive system in the rat (Cechetto and Saper, 1987; Hanamori 2005), posits the pIC as an important site for emotion processing as several physiological changes accompany emotion.

The theory of James-Lange proposes that emotional feelings are the conscious perception of the physiological changes triggered by emotional stimuli (James, 1884). In other words, our nervous system perceives some bodily responses as emotional experiences. These emotional stimuli may be innate or learned releasers. The emotional feelings may result from the representation of these physiological changes in the IC. Thus, the IC appears as a possible site where such autonomic responses and general bodily states are represented consciously as emotional feelings.

Taken together, these data show that the expression of innate freezing is correlated with changes in the activity of a discrete part of the pIC and through electrophysiological single units recordings suggest that the acute change in the FR during freezing behavior appears to be functionally relevant within the innate defense system, stimulating the emergence of an appropriate behavior in response to the predator,

which support the hypothesis that the neuronal activity of the pIC contributes in the expression of the innate fear.

7. CONCLUSIONS

Data presented in this thesis show the relevance of the insular cortex in the expression of freezing, either innate or learned, induced by cat odor. Here, we found that the acute inactivation of the pIC, but not somatosensory cortex, reduced the expression of freezing during a single exposure. The long-term pIC inactivation induced a prolonged and robust reduction in freezing response in subsequent re-exposures to cat odor. By contrast, the reduction in risk assessment behavior lasted no longer than 24 hours after NSTX injection into pIC. Additionally, the conditioning to the context in which rats were exposed to cat odor, was impaired by pIC inactivation with Muscimol, decreasing both freezing as risk assessment behaviors.

We found that cat odor induces an increment in the neuronal activity of the IC, in both pIC and RAIC by positive Fos-ir neurons counting. We also show that freezing response to cat odor stimulus is highly correlated with the expression of this early gene marker.

Our electrophysiological recordings of single-units of the pIC, in living animals, show that neurons modify their FR during innate defensive behaviors (i.e freezing and risk assessment behaviors) relative to non-behavioral periods, in presence of the innate

fear inducer. This pattern of change resulted in a transitory change in the firing rate during the onset of freezing behavior. In contrast, the neuronal activity associated to risk assessment behavior showed a sustained change in the magnitude from the onset of behavior. Interestingly, in our single units' recordings we found three classes of neurons based on statistic and electrophysiological standards. Classification criterion set two populations of pyramidal cells which show differences in the firing pattern and a third group of interneurons selected by waveform of action potential. In addition, we found pIC units that not only responsive specifically during either freezing or risk assessment, but also are responsive during both behaviors.

In summary the present work contributes to the understanding how insula represents and codifies fear and anxiety components in both innate and learned processing, providing data showing for the first time that the neuronal activity of the pIC is important for expressing innate fear and contextual fear and anxiety expressions. These results provide further support for a role of the IC in fear and together with previous findings strongly suggest that the IC may modulates fear responses to innate and learned environmental threats.

8. REFERENCES

Abboud, H., Berroir, S., Labreuche, J., Orjuela, K. and Amarenco, P. (2006). Insular involvement in brain infarction increases risk for cardiac arrhythmia and death. Ann. Neurol. **59**, 691-699.

Acuña-Goycolea, C., Fueltealba, P. and Torrealba, F. (2000). Anatomical substrate for separate processing of ascending and descending visceral information in the nucleus of the solitary tract of the rat. Brain Res. **883**, 229-232.

Aguilar-Rivera, M. (2012). ¿How do we feel?. Role of the insular cortex in the interoception. PhD. thesis of the Physiological Sciences program, Pontificia Universidad Catolica de Chile.

Allen, G.V., Saper, C.B., Hurley, K.M., Cechetto, D.F. (1991). Organization of visceral and limbic connections in the insular cortex of the rat. J. Comp. Neurol. **311**, 1-16.

Alvarez, R.P., Biggs, A., Chen, G., Pine, D.S. and Grillon, C. (2008). Contextual fear conditioning in humans: cortical-hippocampal and amygdala contributions. J. Neurosci. **28**, 6211-6219.

Alvarez, R.P., Chen, G., Bodurka, J., Kaplan, R., Grillon, C. (2011). Phasic and sustained fear in humans elicits distinct patterns of brain activity. Neuroimage. **55**, 389-400.

Alves, F.H., Gomes, F.V., Reis, D.G., Crestani, C.C., Corrêa, F.M., Guimarães, F.S. and Resstel, L.B. (2013). Involvement of the insular cortex in the consolidation and expression of contextual fear conditioning, Eur. J. Neurosci. **38**, 2300-2307.

Apfelbach, R., Blanchard, C.D., Blanchard, R.J., Hayes, R.A. and McGregor, I.S. (2005). The effects of predator odors in mammalian prey species: a review of field and laboratory studies. Neurosci. Biobehav. Rev. **29**, 1123-1144.

Ardid, S., Vinck, M., Kaping, D., Marquez, S., Everling, S. and Womelsdorf, T. (2015) Mapping of functionally characterized cell classes onto canonical circuit operations in primate prefrontal cortex. J Neurosci. **35**:2975-2991.

Bailey, P. and Bremer, F. (1938). A sensory cortical representation of the vagus nerve with a note on the effects of low blood pressure on the cortical electrogram. J. Neurophysiol. **1**, 405-412.

Barrett, L.F. (2006). Are emotions natural kinds? Perspect. Psychol. Sci. **1**, 28-50.

Blanchard, D.C. and Blanchard, R.J. (1972). Innate and conditioned reactions to threat in rats with amygdaloid lesions. J. Comp. Physiol. Psychol. **81**, 281-290.

Blanchard, R.J. and Blanchard, D.C. (1989). Attack and defense in rodents as ethoexperimental models for the study of emotion. Prog Neuropsychopharmacol Biol Psychiatry. **13**(Suppl): S3-S14.

Blanchard, D.C., Blanchard, R.J. and Rodgers, R.J. (1991). Risk assessment and animal models of anxiety. In: Olivier, B., Mos, J., Slangen, J.L. (Eds.), Animal Models in Psychopharmacology. Birkhauser Verlag AG, Basel, pp. 117–134.

Blanchard, R.J., Nikulina, J.N., Sakai, R.R., McKittrick, C., McEwen, B. and Blanchard, D.C. (1998). Behavioral and endocrine changes following chronic predatory stress. Physiol. Behav. **63**, 561-569.

Blanchard, R.J., Yang, M., Li, C.I., Gervacio, A. and Blanchard, D.C. (2001). Cue and context conditioning of defensive behaviors to cat odor stimuli. Neurosci. Biobehav. Rev. **25**, 587-595.

Blanchard, D. C., Canteras, N. S., Markham, C. M., Pentkowski, N. S. and Blanchard, R. J. (2005). Lesions of structures showing FOS expression to cat presentation: effects on responsivity to a cat, cat odor, and nonpredator threat. Neurosci. Biobehav. Rev. **29**, 1243-1253.

Blanchard, D.C. and Blanchard, R.J. (2008). Defensive behaviors, fear and anxiety. In Handbook of anxiety and fear (Blanchard RJ, Blanchard DC, Griebel G and Nutt DJ, ed), pp63-76. Amsterdam: Elsevier Academic Press.

Blanchard, D.C., Griebelb, G., Pobbec, R. and Blanchard, R.J. (2011). Risk assessment as an evolved threat detection and analysis process. Brain Res. **35**, 991-998.

Brinkmann, L., Buff, C., Feldker, K., Tupak, S.V., Becker, M.P.I., Herrmann, M.J. and Straube, T. (2017). Distinct phasic and sustained brain responses and connectivity of amygdala and bed nucleus of the stria terminalis during threat anticipation in panic disorder. Psychol Med, **47**, 2675-2688.

Cacioppo, J.T., Berntson, G.G., Larsen, J.T., Poehlmann, K.M., Ito, T.A. (2000). The psychophysiology of emotion. In: Lewis R, Haviland-Jones JM (Eds.). The Handbook of Emotion. 2nd ed. Guilford Press, New York, pp. 173-191.

Calder, A.J., Keane, J., Manes, F., Antoun, N. and Young, A.W. (2000). Impaired recognition and experience of disgust following brain injury. Nat. Neurosci. **3**, 1077-1078.

Canteras, N.S., Simerly, R.B. and Swanson, L.W. (1994). Organization of projections from the ventromedial nucleus of the hypothalamus: a Phaseolus vulgaris-leucoagglutinin study in the rat. J. Comp. Neurol. **348**, 41-79.

Canteras, N.S., Simerly, R.B. and Swanson, L.W. (1995). Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat. J. Comp. Neurol. **360**, 213-245.

Caruana, F., Jezzini, A., Sbriscia-Fioretti, B., Rizzolatti, G. and Gallese, V. (2011). Emotional and social behaviors elicited by electrical stimulation of the insula in the macaque monkey. Curr. Biol. **21**, 195-199.

Casanova, J.P (2016). Role of the interoceptive insula in the regulation of fear memory. PhD. thesis of the Physiological Sciences program, Pontificia Universidad Catolica de Chile.

Casanova, J.P., Madrid, C., Contreras, M., Rodriguez, M.A., Vazquez, M. and Torrealba, F. (2016). A role for the interoceptive insular cortex in the consolidation of learned fear. Behav. Brain Res. **296**, 70-77.

Cauda, F., Torta, D.M., D'Agata. F., Duca,S., Geminiani, G., Fox, P.T. and Vercelli, A. (2012). Meta-analytic clustering of the insular cortex: characterizing the meta- analytic connectivity of the insula when involved in active tasks. NeuroImage. **62**, 343-355.

Cechetto, D.F. and Chen, S.J. (1990). Subcortical sites mediating sympathetic responses from insular cortex in rats. Am. J. Physiol. **258**, 245-255.

Cechetto, D.F. and Saper, C.B. (1987). Evidence for a viscerotopic sensory representation in the cortex and thalamus in the rat. J. Comp. Neurol. **262**, 27-45.

Chaudhuri, A and Zangenehpour, S. (2002). Chapter V Molecular Activity Maps of Sensory Function. In Handbook of Chemical Neuroanatomy **19**, 103-145.

Choy, K.H.C., Yu, J., Hawkes, D. and Mayorov, D.N. (2012). Analysis of vigilant scanning behavior in mice using two-point digital video tracking. Psychopharmacol. Berl. **221**, 649-657.

Claps, A. and Torrealba, F. (1988). The carotid body connections: a WGA-HRP study in the cat. Brain Res. **455**, 123-133.

Contreras, M., Ceric, F. and Torrealba, F. (2007). Inactivation of the interoceptive insula disrupts drug craving and malaise induced by lithium. Science*.* **318**, 655-658.

Contreras, M., Billeke, P., Vicencio, S., Madrid, C., Perdomo, G., Gonzalez, M. and Torrealba, F. (2012). A role for the insular cortex in long-term memory for context-evoked drug craving in rats*.* Neuropsychopharmacology. **37**, 2101-2108.

Craig, A.D. (2002). How do you feel? Interoception: the sense of the physiological condition of the body. Nat. Rev. Neurosci*.* **3**, 655-666.

Craig, A.D. (2003). Interoception: the sense of the physiological condition of the body. Curr. Opin. Neurobiol. **13**, 500-505.

Craig, A.D. (2009). How do you feel-now? The anterior insula and human awareness. *Nat. Rev. Neurosci.* **10**, 59-70.

Critchley, H.D., Wiens, S., Rotshtein, P., Öhman, A. and Dolan, R.J. (2004). Neuronal systems supporting interoceptive awareness. Nat. Neurosci*.* **7**, 189-195.

Critchley, H.D. and Harrison, N.A. (2013). Visceral influences on brain and behavior. Neuron. **77**, 624-638.

Damasio, A.R (1994). Descartes' error: emotion, reason, and the human brain. New York: Grosset Putnam.

Damasio, A.R. (1998). The somatic marker hypothesis and the possible functions of the prefrontal cortex. In A. C. Roberts, T. W. Robbins, and L. Weiskrantz (Eds.). The prefrontal cortex, executive and cognitive functions. Nueva York: Oxford University Press, pp. 36-50.

Damasio, A.R., Grabowski, T.J., Bechara, A., Damasio, H., Ponto, L.L. and Parvizi, J. (2000). Subcortical and cortical brain activity during the feeling of self-generated emotions. Nat. Neurosci. **3**, 1049-1056.

Damasio, A.R. and Carvalho, G.B. (2013). The nature of feelings: evolutionary and neurobiological origins. Nat. Rev. Neurosci. **14**,143-152.

Darwin, C. (1872). The Expression of Emotion in Man and Animals. University of Chicago Press, Chicago.

Dell, P. and Olson, R. (1951). Thalamic, cortical and cerebella projections of vagal visceral afferents. C. R. Seances Soc. Biol. Fil. **145**,1084-1088.

Deng, H., Xiao, X. and Wang, Z. (2016). Periaqueductal gray neuronal activities underlie different aspects of defensive behaviors. J. Neurosci. **36**, 7580-7588.

Dielenberg, R.A. and McGregor, I.S. (1999). Habituation of the hiding response to cat odor in rats (Rattus norvegicus). J. Comp. Psychol. **113**, 376-387.

Dielenberg, R.A. and McGregor, I.S. (2001) Defensive behavior in rats predatory odors: a review. Neurosci. Biobehav. Rev. **25**, 597-609.

Dielenberg, R.A. Carrive, P. and McGregor, I.S. (2001) a. The cardiovascular and behavioral response to cat odor in rats: unconditioned and conditioned effects. Behav. Brain Res. **897**, 228-237.

Dielenberg, R.A. Hunt, G.E. and McGregor, I.S. (2001) b. When a rat smells a cat: the distribution of Fos immunoreactivity in rat brain following exposure to a predatory odor. Neurosci. **104**, 1085-1097.

Dielenberg, R.A., Leman, S. and Carrive, P. (2004). Effect of dorsal periaqueductal gray lesions on cardiovascular and behavioral responses to cat odor exposure in rats. Behav. Brain Res. **153**, 487-496

Ekman, P., Levenson, R.W. and Friesen, W.V. (1983). Autonomic nervous system activity distinguishes among emotions. Science. **221**,1208-1210.

Ekman, P. (1994). All emotions are basic. In: Davidson, R.J., Ekman, P., Scherer, K. (Series Eds.), Ekman, P., Davidson, R.J. (Vol. Eds.), The Nature of Emotion: Fundamental Questions. Oxford, New York, pp. 15-19.

Ekman, P. (2007). Emotions revealed: Recognizing faces and feelings to improve communication and emotional life. New York: Henry Holt.

Epstein-Barash, H., Shichor, I., Kwon, A.H., Hall, S., Lawlor, M.W., Langer, R. and Kohane, D.S.P. (2009). Prolonged duration local anesthesia with minimal toxicity. Proc Natl Acad Sci USA **106**, 7125-7130.

Falkner, A. L. Falkner1, Logan Grosenick, L., Davidson, T. J., Deisseroth, K. and Lin, D. (2016). Hypothalamic control of male aggression-seeking behavior. Nat. Neurosci. **19**, 596-604.

Feinstein, J.S., Buzza, C., Hurlemann, R., Follmer, R.L., Dahdaleh, N.S., Coryell, W.H., Welsh, M.J., Tranel, D., Wemmie, J.A. (2013). Fear and panic in humans with bilateral amygdala damage. Nat. Neurosci. **16**, 270-272.

Figueiredo, H.F., Bodie, B.L., Tauchi, M., Dolgas, C.M. and Herman, J.P. (2003). Stress integration after acute and chronic predator stress: differential activation of central stress circuitry and sensitization of the hypothalamo–pituitary–adrenocortical axis. Endocrinol.**144**, 5249-5258.

Flack, W.F., Laird, J.D. and Cavallaro, L. (1999). Separate and combined effects of facial expressions and bodily postures on emotional feelings. Eur. J. Soc. Psychol*.* **29**, 203-217.

Friedman, B.H. (2010). Feeling and the body: The Jamesian perspective on autonomic specificity of emotion. Biol. Psychol. **84**, 383-393.

Garfinkel, S.N., Minati, L., Gray, M.A., Seth, A.K., Dolan, R.J. and Critchley, H.D. (2014). Fear from the heart: sensitivity to fear stimuli depends on individual heartbeats. J. Neurosci. **34**, 6573-6582.

Garfinkel, S.N. and Critchley, H.D. (2016). Threat and the body: how the heart supports fear processing. Trends Cogn. Sci. **20**, 34-46.

Gray, C.M., Maldonado, P.E., Wilson, M and McNaughton, B. (1995). Tetrodes markedly improve the reliability and yield of multiple single-unit isolation from multi-unit recordings in cat striate cortex. J Neurosci Methods. **63**, 43-54.

Gross, C.T. and Canteras, N.S. (2012). The many paths to fear. Nat. Rev. Neurosci. **13**, 651-658.

Harris, K.D., Henze, D.A., Csicsvari, J., Hirase, H., Buzsaki, G. (2000). Accuracy of tetrode spike separation as determined by simultaneous intracellular and extracellular measurements. J. Neurophysiol. **84**, 401-414.

Hanamori, T. (2005). Fluctuations of the spontaneous discharge in the posterior insular cortex neurons are associated with changes in the cardiovascular system in rats. Brain Res. **1042**, 82-91.

Hassanpour, M.S., Simmons, W.K., Feinstein, J.S., Luo, Q., Lapidus, R., Bodurka, J., ... and Khalsa, S.S. (2017). The Insular Cortex Dynamically Maps Changes in Cardiorespiratory Interoception. Neuropsychopharmacology. In press.

James, W. (1884). What is an emotion?. Mind. **9**, 188-205.

Jasmin, L., Burkey, A.R., Granato, A., Ohara, P.T. (2004). Rostral agranular insular cortex and pain areas of the central nervous system: a tract-tracing study in the rat. J. Comp. Neurol. **468**, 425-440.

Jordan, D. (1990). Autonomic changes in affective behavior. “Central Regulation of Autonomic Function” (A. D. Loewy and K. M. Spyer, eds.), pp. 349-366. Oxford Univ. Press, NewYork.

Kadir S. N., Goodman D. F. and Harris K. D. (2014). High-dimensional cluster analysis with the masked em algorithm. Neural Comput. **26**, 2379-2394.

Khalsa, S.S., Feinstein, J.S., Wi, L., Feusner, J.D., Adolphs, R. and Hurlemann, R. (2016). Panic anxiety in humans with bilateral amygdala lesions: pharmacological induction via cardiorespiratory interoceptive pathways. J. Neurosci. **36**, 3559-3566.

Klarer, M., Arnold, M., Günther, L., Winter, C., Langhans, W. and Meyer, U. (2014) Gut vagal afferents differentially modulate innate anxiety and learned fear. J. Neurosci. **34**, 7067-7076.

Krupa, D.J., Ghazanfar, A.A. and Nicolelis, M.A. (1999). Immediate thalamic sensory plasticity depends on corticothalamic feedback, Proc Natl Acad Sci U S A **96**, 8200-8205.

Kunwar, P.S., Zelikowsky M., Remedios R., Cai H., Yilmaz M., Meister M. and Anderson D.J. (2015). Ventromedial hypothalamic neurons control a defensive emotion state. Elife **4**. 1-30.

Kurth, F., Zilles, K., Fox, P.T., Laird, A.R. and Eickhoff, S.B. (2010). A link between the systems: functional differentiation and integration within the human insula revealed by meta-analysis. Brain Struct. Funct. **214**, 519-534.

Kusumoto-Yoshida, I., Liu, H., Chen, B.T., Fontanini, A. and Bonci, A. (2015). Central role for the insular cortex in mediating conditioned responses to anticipatory cues. Proc. Natl. Acad. Sci. USA.**112**, 1190-1195.

Laowattana, S., Zeger, S.L., Lima, J.A., Goodman, S.N., Wittstein, I.S. and Oppenheimer, S.M. (2006). Left insular stroke is associated with adverse cardiac outcome. Neurol. **66**, 477-483.

Lazarus, R.S. (1994). *Emotion and Adaptation*, Oxford University Press.

LeDoux, J.E., Iwata, J., Cicchetti, P. and Reis, D.J. (1988). Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. J. Neurosci. **8**, 2517-2529.

Levenson, R.W. (1992). Autonomic nervous system differences among emotions. Psychological Science. **3**, 23-27.

Li, C.I., Maglinao, T.L. and Takahashi, L.K. (2004). Medial amygdala modulation of predator odor-induced unconditioned fear in the rat. Behav. Neurosci. **118**,324-332.

Lin, D., Boyle, M.P., Dollar, P., Lee, H., Lein, E.S., Perona, P. and Anderson, D.J. (2011). Functional identification of an aggression locus in the mouse hypothalamus. Nature. **470**, 221-226.

Li, H., Chen, L., Li, P., Wang, X. and Zhai, H. (2014). Insular muscarinic signaling regulates anxiety-like behaviors in rats on the elevated plus-maze. Behav. Brain Res. **270**, 256-260.

Luo, Q., Cheng, X., Holroyd, T., Xu, D., Carver, F. W., and Blair, J. (2013). Theta band activity in response to emotional expressions and its relationship with gamma band activity as revealed by MEG and advanced beamformer source imaging. Front. Hum. Neurosci. **7**, 940-945.

Maier, J.X., Wachowiak, M. and Katz, D.B. (2013). Chemosensory convergence on primary olfactory cortex. J. Neurosci. **32**, 17037-17047.

Madrid, C. (2014). A role of the interoceptive system in the conditioned fear. PhD. thesis of the Physiological Sciences program, Pontificia Universidad Catolica de Chile.

Maris, E., and Oostenveld, R. (2007). Nonparametric statistical testing of EEG and MEG data. J. Neurosci. Methods. **164**,177-190.

Martinez, R.C., Carvalho-Netto, E.F., Ribeiro-Barbosa, E.R., Baldo, M.V. and Canteras, N.S. (2011). Amygdalar roles during exposure to a live predator and to a predator-associated context. Neuroscience. **172**, 314-328.

McNaughton, N. and Corr, P.J. (2004). A two-dimensional neuropsychology of defense: fear/anxiety and defensive distance. Neurosci. Biobehav. Rev. **28**, 285-305.

McGregor, I.S. and Dielenberg, R.A. (1999). Differential anxiolytic efficacy of a benzodiazepine on first versus second exposure to a predatory odor in rats. Psycopharmacology. **147**, 174-181.

McGregor, I.S., Hargreaves, G.A., Apfelbach, R. and Hunt, G.E. (2004). Neuronal correlates of cat odor-induced anxiety in rats: region-specific effects of the benzodiazepine midazolam. J. Neurosci. **24**, 4134-4144.

Menon, V. and Uddin, L.Q. (2010). Saliency, switching, attention and control: a network model of insula function. Brain Struct. Funct. **214**, 655-667.

Mesulam, M.M. and Mufson, E.J. (1985). The Insula of Reil in Man and Monkey: Architectonics, connectivity, and Function. In: Cerebral Cortex (Peters A and Jones EG, ed), pp179-223. New York: Plenum Press.

Morris, J.S. and Dolan, R.J. (2004). Dissociable amygdala and orbitofrontal responses during reversal fear conditioning. Neuroimage. **22**, 372-380.

Müller, M. and Fendt, M. (2006). Temporary inactivation of the medial and basolateral amygdala differentially affects TMT-induced fear behavior in rats. Behav. Brain Res. **167**, 57-62.

Muñoz-Abellán, C., Andero, R., Nadal, R., Armario, A. (2008). Marked dissociation between hypothalamic-pituitary-adrenal activation and long-term behavioral effects in rats exposed to immobilization or cat odor. Psychoneuroendocrinology. **33**, 1139-1150.

Oppenheimer, S.M., Wilson, J.X., Guiraudon, C. and Cechetto, D.F. (1991). Insular cortex stimulation produces lethal cardiac arrhythmias: a mechanism of sudden death. Brain Res. **550**, 115-121.

Papes, F., Logan, D.W. and Stowers, L. (2010). The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. Cell. **141**, 692-703.

Paulus, M.P. and Stein, M.B. (2010). Interoception in anxiety and depression. Brain Struct. Funct. **214**, 451-463.

Penfield, W. and Rasmussen, T. (1950). The cerebral cortex of man. A clinical study of localization of function. New York: Macmillan.

Penfield, W. and Faulk, M. Jr. (1955). The insula, further observations on its function. Brain. **78**, 445-470.

Pérez-Gómez, A., Bleymehl, K., Stein, B., Pyrski, M., Birnbaumer, L., Munger, S.D., Leinders-Zuffall, T., Zuffal, F. and Chamero, P. (2015). Innate predator odor aversion driven by parallel olfactory subsystems that converge in the ventromedial hypothalamus. Curr. Biol. **25**, 1340-1346.

Phelps, E.A., O'Connor, K.J., Gatenby, J.C., Gore, J.C., Grillon, C. and Davis, M. (2001). Activation of the left amygdala to a cognitive representation of fear. Nat. Neurosci. **4**, 437-441.

Quirk, M.C., Sosulski, D.L., Feierstein, C.E., Uchida, N., Mainen, Z.F. (2009). A defined network of fast-spiking interneurons in orbitofrontal cortex: responses to behavioral contingencies and ketamine administration. Front. Syst. Neurosci. **3**, 1-13.

Reed, M.D., Price, K.E., Archbold, J., Moffa, A. and Febo, M. (2013). Predator odor-evoked BOLD activation in the awake rat: modulation by oxytocin and V(1)a vasopressin receptor antagonists. Brain Res. **1494**, 70-83.

Reil, J. (1809). Die sylvische Grube. Arch. physiol. **9**, 195-208.

Rolls, E.T., Kringelbach, M.L. and De Araujo, I.E.T. (2003). Different representations of pleasant and unpleasant odours in the human brain. Eur. J. Neurosci. **18**, 695-703.

Ruggiero, D.A. Mraovitch, S., Granata, A.R., Anwar, M. and Reis, D.J. (1987). A role of insular cortex in cardiovascular function. J. Comp. Neurol. **257**, 189–207.

Saper, C.B. (1982). Convergence of autonomic and limbic connections in the insular cortex of the rat. J. Comp. Neurol. **210**,163-173.

Saper, C.B. (2002). The central autonomic nervous system: conscious visceral perception and autonomic pattern generation. Annu. Rev. Neurosci. **25**, 433-469.

Samuelsen, C. and Fontanini A. (2017) Processing of intraoral olfactory and gustatory signals in the gustatory cortex of awake rats. J. Neurosci. **37**, 244-257.

Shi, C.J. and Cassell, M.D. (1998). Cortical, thalamic, and amygdaloid connections of the anterior and posterior insular cortices. J. Comp. Neurol. **399**, 440-468.

Shi, C. and Davis, M. (1999). Pain pathways involved in fear conditioning measured with fear-potentiated startle: Lesion studies. J Neurosci. **19**, 420-430.

Shipley, M.T. and Geinisman, Y. (1984). Anatomical evidence for convergence of olfactory, gustatory, and visceral afferent pathways in mouse cerebral cortex. Brain Res. Bull. **12**, 221-226.

Silva, B.A., Mattucci, C., Krzywkowski, P., Murana, E., Illarionova, A., Grinevich, V., Canteras, N.S., Ragozzino, D. and Gross, C.T. (2013). Independent hypothalamic circuits for social and predator fear. Nat. Neurosci. **16**, 1731-1733.

Silva, B.A, Gross, C.T. and Gräff, J. (2016). The neuronal circuits of innate fear: detection, integration, action, and memorization. Learn Mem. **23**, 544-555

Simmons, A.N., Stein, M.B., Strigo, I.A., Arce, E., Hitchcock, C., Paulus, M.P. (2011). Anxiety positive subjects show altered processing in the anterior insula during anticipation of negative stimuli. Hum. Brain Mapp. **32**,1836-1846

Stapples, L.G., Hunt G.E., Cornisha, J.L., McGregor, I.S. (2005). Neuronal activation during cat odor-induced conditioned fear and 'trial 2' fear in rats. Neurosci. Biobehav. Rev. **29**, 1265-1277.

Steenland, H.W. and Zhuo, M. (2009). Neck electromyography is an effective measure of fear behavior. J. Neurosci. Methods, **177**, 355-360.

Stemmler, G. (2004). Physiological processes during emotion. In: Philippot, P., Feldman, R.S. (Eds.), The Regulation of Emotion. Erlbaum, Mahwah, NJ, pp. 33-70.

Stujenske JM, Likhtik E, Topiwala MA and Gordon JA (2014) Fear and safety engage competing patterns of theta-gamma coupling in the basolateral amygdala. Neuron **83**, 919-933

Swanson, L.W. (1998). Brain Maps: Structure of the Rat Brain. (Elsevier Science BV, Amsterdam).

Takahashi, L., Nakashima, B., Hong, H. and Watanabe, K. (2005). The smell of danger: a behavioral and neuronal analysis of predator odor-induced fear. Neurosci. Biobehav. Rev. **29**, 1157-1167.

Torrealba, F. and Claps, A. (1988). The vagal connection of the carotid sinus. Neurosci. Lett. **93**, 186-190.

Vianna, D. and Carrive, P. (2005). Changes in cutaneous and body temperature during and after conditioned fear to context in the rat. Eur. J. Neurosci. **21**, 2505-2512.

Wang, L., Chen, I. and Lin, D. (2015). Collateral pathways from the ventromedial hypothalamus mediate defensive behaviors. Neuron. **85**, 1344-1358.

Yasui, Y., Breder, C., Saper, C. and Cechetto, D. (1991). Autonomic responses and efferent pathways from the insular cortex in the rat. J. Comp. Neurol. **303**, 355-374.

Zangrossi, H. and File, S.E. (1992). Behavioral consequences in animal tests of anxiety and exploration of exposure to cat odor. Brain Res. Bull. **29**, 381-388.

Zhang, Z. and Oppenheimer, S. (2000). Baroreceptive and somatosensory convergent thalamic neurons project to the posterior insular cortex in the rat. Brain. Res. **861**, 241-256.

9. APPENDIX

TABLE Nº1: Changes in appearance and ANS activity associated to fear.

Type	Change	ANS-Mediated Basis
Coloration	**Blanching**	**Vasoconstriction** (Levenson RW, 2003)
Moisture and secretions	**Sweating** **Clamminess**	**Sweat glands** (Levenson RW, 2003)
Protrusions	**Piloerection**	**Muscle fibers at base of hair follicle** (Levenson RW, 2003)
Apparence of eye	**Dilation** **Bulging**	**Pupils, Eyelid muscle** (Levenson RW, 2003)
Heart Rate	**Increases**	**Vasocontriction** (Cacciopo JT, et al.,1993)
Blood pressure	**Increases**	**Vasocontriction** (Cacciopo JT, et al.,1993)
Respiration Rate	**Increases**	**Vasocontriction** (Cacciopo JT, et al.,1993)
Muscle	**Tension**	**Contriction** (Jordan D., 1990)
Muscle blood flow	**Increases**	**Hypotension** (Jordan D., 1990)

TABLE Nº2: Summary of changes in the FR of pIC single-units induced by cat odor.

pIC neurons	LFR	HFR	INeu
Excited	36 (41)	53 (8)	25 (3)
Inhibited	19 (22)	7 (1)	33 (4)
No changes	45 (51)	40 (6)	42 (5)
Total	100 (114)	100 (15)	100 (12)

TABLE Nº3: Percentage of cells excited or inhibited for all neurons recorded during freezing, risk assessment or both.

pIC neurons	Freezing	Freezing/Risk Assessment	Risk Assessment
Excited	11% (16)	11% (16)	14% (20)
Inhibited	7% (9)	6% (8)	7% (10)

TABLE Nº4: Percentage of responsiveness of a subtype of pyramidal neurons (LFR) during innate defensive behaviors.

LFR (n=114)	Freezing	Freezing/Risk Assessment	Risk Assessment
Excited	13% (15)	11% (11)	12% (15)
Inhibited	6% (7)	6% (7)	7% (8)

TABLE Nº5: Percentage of responsiveness of a subtype of pyramidal neurons (HFR) during innate defensive behaviors.

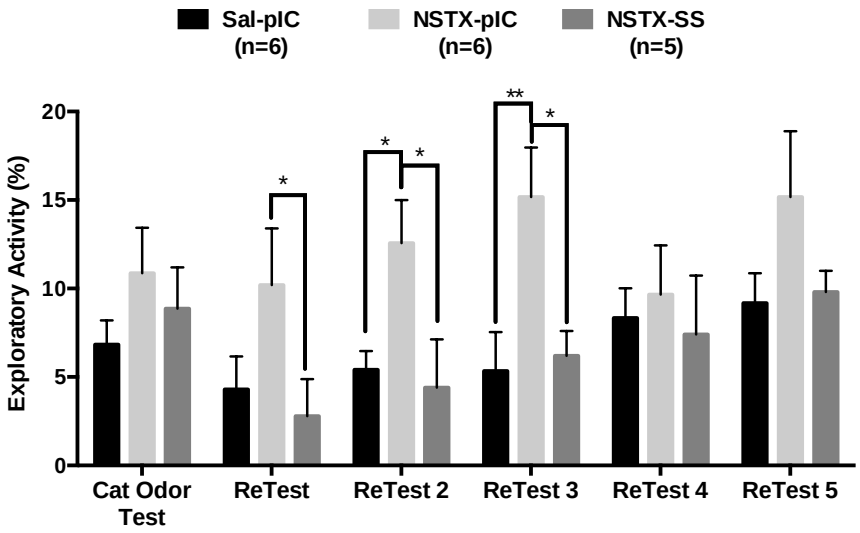
HFR (n=15)	Freezing	Freezing/Risk Assessment	Risk Assessment
Excited	0	27% (4)	27% (4)
Inhibited	7% (1)	0	0

TABLE Nº6: Percentage of responsiveness of interneurons (INeu) during innate defensive behaviors

INeu (n=12)	Freezing	Freezing/Risk Assessment	Risk Assessment
Excited	8% (1)	8% (1)	8% (1)
Inhibited	8% (1)	8% (1)	16% (2)

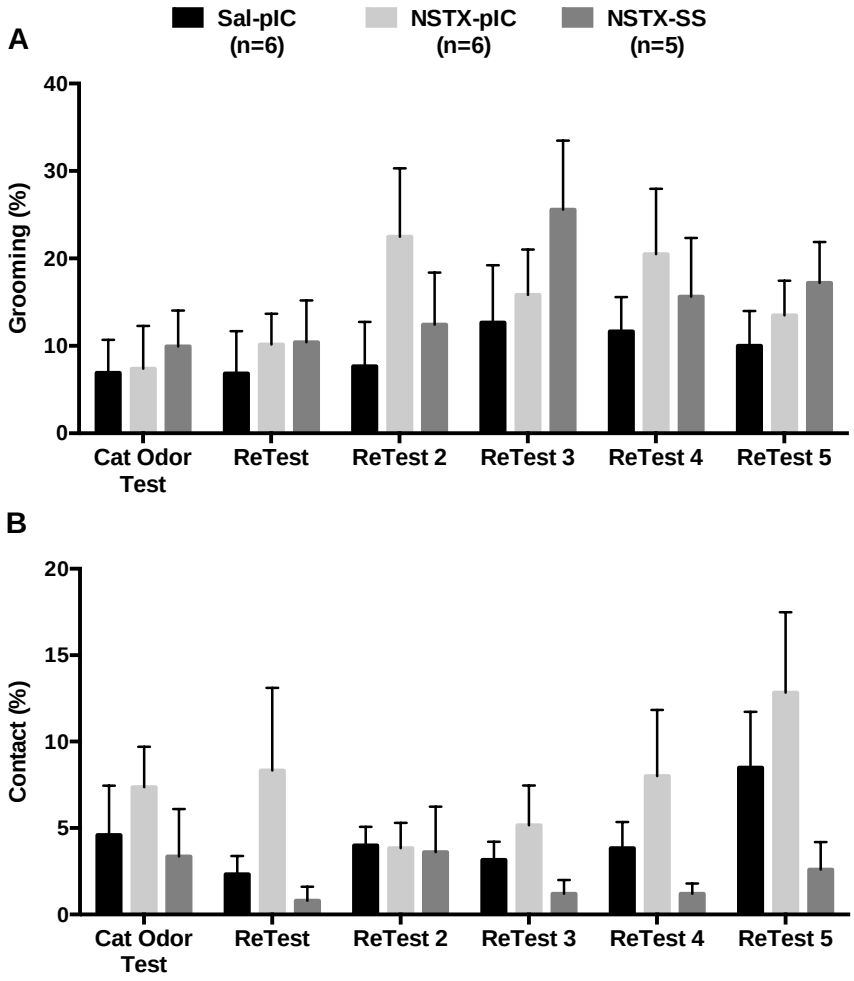
6. RESULTS

6.1.4 Long-term pIC inactivation reduces fear response in response to consecutive cat odor exposures.



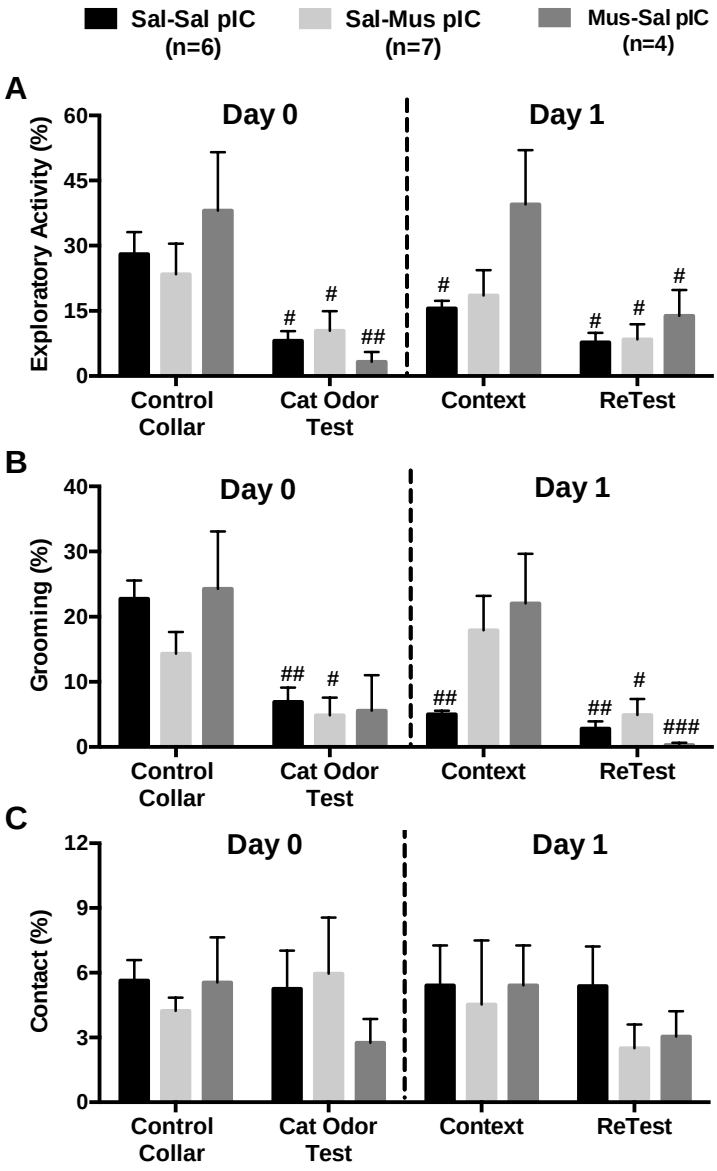
SUPPLEMENTARY Nº1. The long-term pIC inactivation induced an increment of exploratory activity. Immediately after first exposure to cat odor, rats were infused once with saline or Neosaxitoxin (NSTX) into the pIC. Another group was infused once with NSTX into the SS. In the subsequent tests (ReTest [day 1], ReTest 2[day 2], ReTest 3[day 4], ReTest 4 [day14] and ReTest 5 [day 29]) they were re-exposed to cat odor and the behavioral responses recorded. The bars show the time spent in exploratory activity. *p<0.05, **p<0.01; comparisons between groups. Two-way ANOVA followed by Bonferroni test. All values are expressed as mean + SEM.

6.1.4 Long-term pIC inactivation reduces fear response in response to consecutive cat odor exposures.



SUPPLEMENTARY Nº2. The long-term pIC inactivation had no effect on non-defensive behaviors. Immediately after first exposure to cat odor, rats were infused once with saline or Neosaxitoxin (NSTX) into the pIC. Another group was infused once with NSTX into the SS. In the subsequent tests (ReTest [day 1], ReTest 2[day 2], ReTest 3[day 4], ReTest 4 [day14] and ReTest 5 [day 29]) they were re-exposed to cat odor and the behavioral responses recorded. A-B. The bars show the time spent in grooming and contact behaviors, respectively. *p<0.05, **p<0.01; comparisons between groups. Two-way ANOVA followed by Bonferroni test. All values are expressed as mean + SEM.

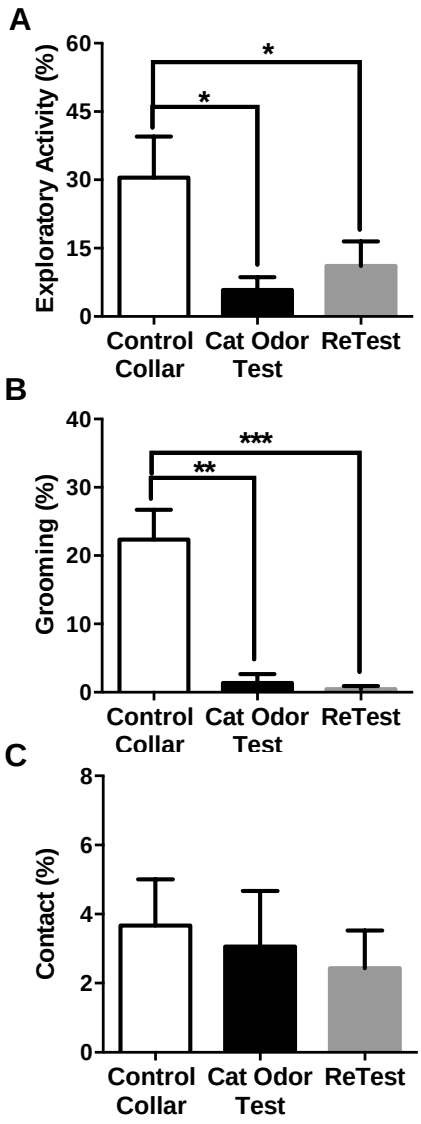
6.1.5. Evaluate the effect of pIC inactivation on context using Muscimol, a GABAA-R agonist in rats exposed to cat odor.



SUPPLEMENTARY Nº3. Short-term pIC inactivation has not an effect on non-defensive behaviors during context exposure. Rats received saline (n=13) or Muscimol (n=4) into the pIC thirty minutes before Cat Odor Test (Day 0). Next day, rats that had received saline previous day, they received saline (n=6) or Muscimol (n=7) thirty minutes before context exposure. As well as rats that received Muscimol previous day, they received saline (n=4) before context exposure. A-C. The bars show the percentage of time spent in each non-defensive behavior: exploratory activity, grooming and contact (continuation page 142).

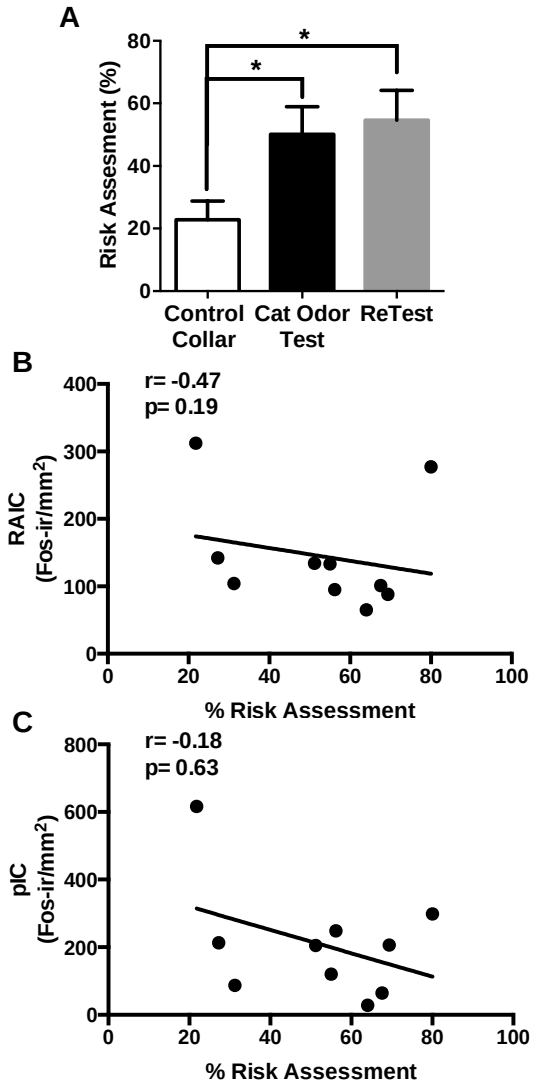
Note that Muscimol-pIC rats had no effect on non-defensive behaviors during context exposure. *p<0.05, **p<0.01; comparisons between groups. Two-way ANOVA followed by Bonferroni test. All values are expressed as mean + SEM.

6.2.1 Determine whether the activity of pIC neurons correlates with innate fear expression.



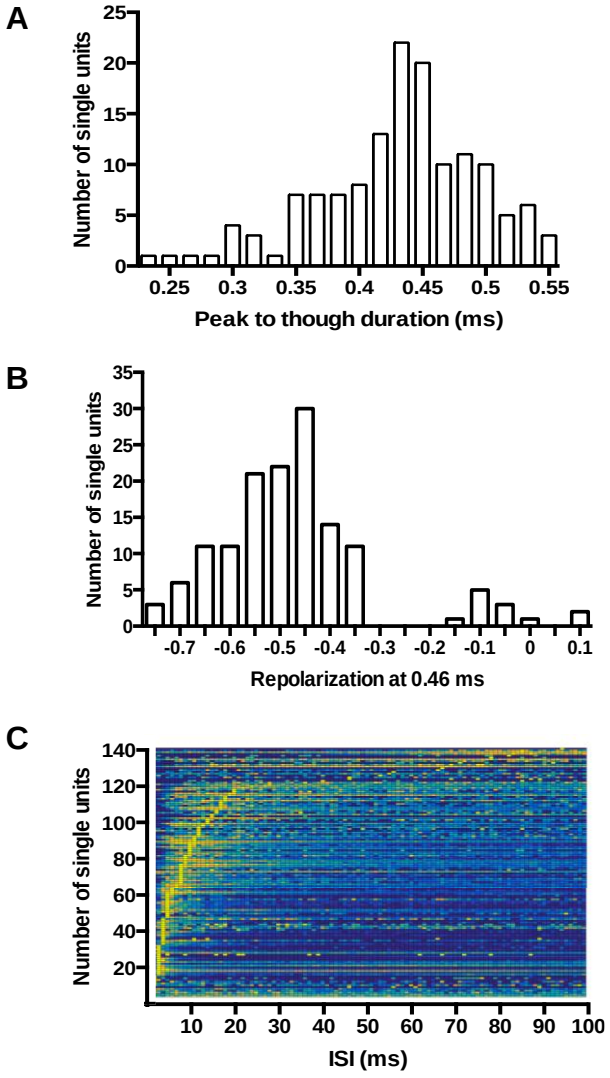
SUPPLEMENTARY Nº4. Behavioral pattern of non-defensive responses to Cat Odor Test and Retest. A-C showing % of the exploratory activity, grooming and contact behaviors in response to control collar (unworn familiar collar), cat odor test and retest. One-way ANOVA followed by Tukey test for each behavior, *p<0.05, comparisons relative to control collar.

6.2.2 Determine whether the activity of pIC neurons correlate with innate fear expression.



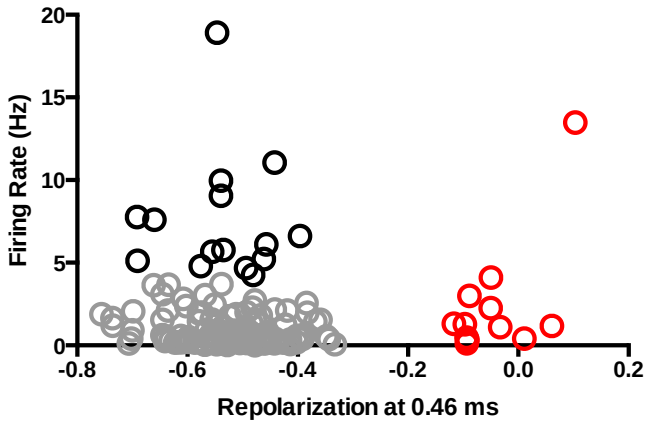
SUPPLEMENTARY Nº5. The neuronal activity of the IC is not correlated with risk assessment behavior. A. Anxiety expression in cat odor test or retest showed higher level of risk assessment relative to rats exposed to the control collar. B-C. Correlations between % of risk assessment over total time and number of Fos-ir neurons in different IC regions (RAIC and pIC). The rats were exposed to cat odor for the first time (Cat odor test) or cat odor for the second time (cat odor retest). Correlation value and significance see in inset. *p<0.05. One-way ANOVA followed by Tukey test for risk assessment (A) and Spearman rank order correlation (B-C).

6.3.1 Evaluate the single-unit activity in the pIC underlying innate fear expression.



SUPPLEMENTARY Nº6. Two distinct cell types in the pIC of the rat: putative pyramidal neurons and putative interneurons cells. A-B. Illustrations of the two features used to characterize extracellular APs: the peak to trough duration (A) and the time for repolarization (B). C. ISI (Inter Spike Interval) histogram for all recorded cells. The data in each row were normalized by dividing by the maximum.

6.3.1 Evaluate the single-unit activity in the pIC underlying innate fear expression.



SUPPLEMENTARY Nº7. Two cell unit's classes of putative pyramidal cells in pIC of the rat. Normalized AP amplitude at 0.46 ms v/s firing rate, revealing three electrophysiological cell classes). K indicated 4.2 Hz. Gray (LFR), black (HFR) and red (INeu) dots. LFR as putative pyramidal cell showing a low firing rate; HFR as putative pyramidal cell showing a high firing rate and Ineu as interneurons.