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Dissertação no âmbito do Mestrado em Biologia Celular e Molecular, com especialização em Neurobiologia orientada pela Professora Doutora Anna Beyeler e pela Professora Doutora Ana Luísa Carvalho apresentada ao Departamaneto de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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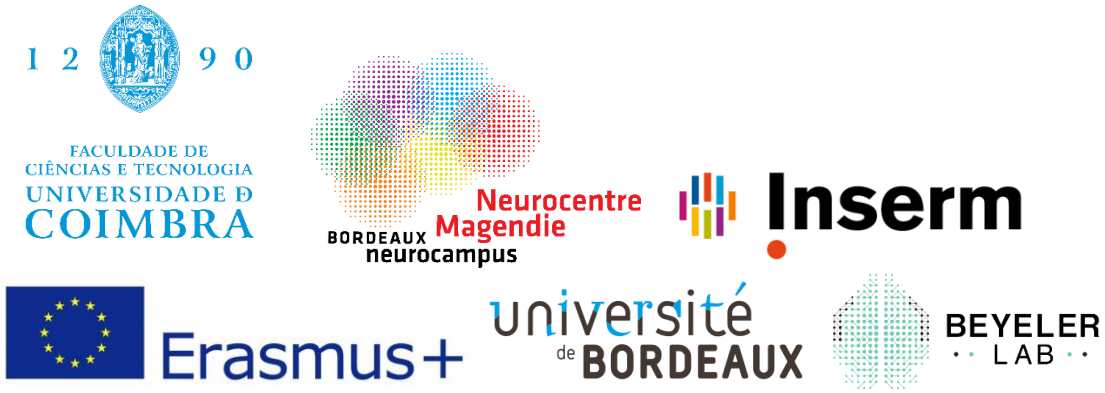
Ana Marta Capaz, Junho 2020

The following work occurred in the Beyeler Lab of the Neurocentre Magendie, located on the Bordeaux Neurocampus (France) and supported by the French National Institute of Health (*Institut National de la Santé et de la Recherche Médicale, INSERM)*. The internship occurred upon agreement between the Faculty of Science and Technology of the University of Coimbra (FCTUC), the University of Bordeaux and INSERM, for Student Exchange Programme Erasmus+.



Ana Marta Capaz, Junho 2020

Para a minha mãe, para o meu pai, e para a Sónia.

Obrigada.

Ana Marta Capaz, Junho 2020

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ABBREVIATIONS

AAV	Adeno Associated Virus
ACC	Anterior Cingulate Cortex
AMPA	Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AP	Anterior / Posterior
CaM	Calmodulin
CaMKIIα	Calcium / Calmodulin-dependent Protein Kinase Type II Subunit Alpha
CeA	Central Amygdala
ChR2	Channelrhodopsin 2
CNQX	Cyanquixaline
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
DV	Dorso / Ventral
EEG	Electroencephalography
EPM	Elevated Plus Maze
EPSC	Excitatory Post Synaptic Current
fMRI	Functional Magnetic Resonance Imaging
GABA	Gamma-Aminobutyric Acid
GAD	Generalized Anxiety Disorder
GFP	Green Fluorescent Protein
ICa	Anterior Insular Cortex
ICp	Posterior Insular Cortex
IPSC	Inhibitory Post Synaptic Current
LH	Lateral Hypothalamus
ML	Medial / Lateral
NMDA	N-Methyl-D-Aspartate
OFT	Open Field Test
OFT-FD	Open Field Test After Food Deprivation
OX	Orexin
PBS	Phosphate-Buffered Saline
PET	Positron Emission Tomography
PFA	Paraformaldehyde
RTPA	Real Time Place Avoidance
RTPP	Real Time Place Preference
SAD	Social Anxiety Disorder
YFP	Yellow Fluorescent Protein

RESUMO

A valência emocional é o valor subjetivo atribuído a estímulos sensoriais. O facto de a capacidade de avaliação da valência estar diminuída em pacientes com patologias de ansiedade sugere fortemente uma sobreposição entre os circuitos neuronais que codificam a ansiedade e a valência. O cortex insular, ou insula, é uma região crucial na codificação da valência no cérebro dos mamíferos, e a sua atividade está aumentada em pacientes com patologias de ansiedade e em indivíduos saudáveis durante situações anxiogénicas. No entanto, ainda é desconhecido como é que a insula controla estas funções, e se existem populações seletivamente envolvidas na valência e/ou ansiedade.

Entender os circuitos neuronais envolvidos na ansiedade, e que estejam alterados em patologias relacionadas pode revelar alvos neuronais específicos para novas abordagens terapêuticas. Neste contexto, decidimos estudar os neurónios que projetam do cortex insular anterior para o hipotálamo lateral (ICa-LH), uma região do cérebro que controla a resposta corporal na ansiedade, como o ritmo respiratório, a pressão sanguínea, e o ritmo cardíaco. Colocámos a hipótese de que os neurónios ICa-LH estariam hiper-ativados em espaços anxiogénicos, em animais saudáveis.

Para testar esta hipótese, utilizámos fotometria de fibra, um método de registo que permite medir a atividade neuronal de populações específicas, em murganhos, durante um conjunto de testes comportamentais relacionados com ansiedade e valência. Realizámos os testes de ansiedade clássicos *elevated plus maze* e *open field test*. Para avaliar a atividade neuronal na presença de estímulos de valência positiva, executámos os testes de alimento, água, e sacarose num novo ambiente. Finalmente, realizámos testes de valência negativa: plataforma quente e *looming*.

Descobrimos que a atividade dos neurónios ICa-LH estava diminuída quando os animais estavam na presença de uma recompensa, mas apenas em espaços anxiogénicos. Isto sugere que esta população participa na codificação da sobreposição entre o instinto de aproximação à recompensa, e o instinto de evasão de um espaço novo (anxiogénico), visto que a sub-ativação ocorre apenas quando ambos os componentes estão presentes. Também descobrimos que a atividade neuronal ICa-LH não estava aumentada em espaços anxiogénicos, nos testes de ansiedade clássicos, sugerindo que estes neurónios não são relevantes para o papel global do cortex insular anterior na ansiedade. Não observámos alterações da atividade nos testes de valência negativa. Visto que poucos dados foram recolhidos, não são suficientes para afirmar que estes neurónios não têm um papel na codificação da valência negativa, em geral. Para melhorar os nossos resultados, precisaríamos de realizar mais experiências utilizando estímulos de valência negativa. De modo a verificar se os neurónios ICa-LH estão causalmente envolvidos no conflito interno entre o instinto de aproximação a um estímulo de valência positiva e o instinto de evasão de um novo espaço, necessitaríamos de realizar experiências de optogenética. Esperamos que a inibição desta população induza um aumento no comportamento de procura de recompensa.

Propomos que os neurónios ICa-LH estão envolvidos no reconhecimento de estímulos de valência positiva, em conjugação com estímulos anxiogénicos. Este contexto comportamental poderá ser um potencial alvo de estudo em condições fisiopatológicas. Podemos postular que os neurónios ICa-LH modulam como os estímulos de valência positiva são percecionados em espaços anxiogénicos, ou que a ansiedade é modulada pela presença de estímulos de valência positiva.

Visto que a ansiedade e a valência têm um papel importante nas interações sociais, também estudámos a população ICa-LH durante observação social. Neurónios espelho são células que disparam quando um indivíduo realiza uma determinada ação, e quando observa outro indivíduo a realizar a mesma ação. Estas células foram identificadas em humanos e macacos, em várias regiões cerebrais, incluindo o cortex insular. No entanto, a existência de atividade de espelho no cérebro de roedores ainda está em debate. Para abordar esta questão, aplicámos um protocolo comportamental de observação social e realizámos registos preliminares. A nossa hipótese era que os neurónios ICa-LH teriam atividade de espelho, mas os nossos resultados preliminares indicaram o contrário. Contudo, para termos a certeza de que esta população não tem atividade de espelho, a experiência deveria ser repetida, idealmente com um método de registo de células individuais.

Este estudo define os neurónios ICa-LH como um possível substrato neuronal para a codificação de estímulos de valência positiva em contextos anxiogénicos, e exclui a possibilidade de que esta população neuronal contribua para a hiper-ativação do cortex insular anterior em situações de elevada ansiedade.

Palavras-chave: Cortex insular, Ansiedade, Valência, Observação social, Fotometria por fibra.

ABSTRACT

Emotional valence is the subjective value assigned to sensory stimuli. The fact that valence assessment is disrupted in anxiety disorders patients strongly suggests an overlap between the neuronal circuits encoding valence and anxiety. The insular cortex, or insula, is a crucial region for valence encoding in the mammalian brain, and its activity is increased in anxiety-disorder patients and in healthy individuals during high-anxiety situations. However, it remains elusive how the insula controls these functions and whether selective populations are involved in valence and/or anxiety.

Understanding the neuronal circuits involved in anxiety and how they are disrupted in animal models of anxiety disorders can provide neuronal targets for new therapeutic approaches. In this regard, we decided to study the neuronal population that projects from the anterior insular cortex to the lateral hypothalamus (ICa-LH), a brain region controlling bodily response in anxiety such as respiratory rate, blood pressure and heart rate. We hypothesized that the ICa-LH neurons are hyper-activated in anxiogenic spaces, in healthy animals.

To test this hypothesis, we used fiber photometry, a recording method allowing to measure the neuronal activity of specific populations in mice, during a range of anxiety- and valence-related behavioral tests. We performed the classical anxiety tests elevated plus maze and open field test. To evaluate the neural activity in the presence of positive valence stimuli, we performed the food, water and sucrose in new environment tests. Finally, we performed the negative valence tests: hot plate and looming.

Interestingly, we found that the activity of ICa-LH neurons was decreased when the animal was in presence of a reward, but only in an anxiogenic space. This suggests that this population participates to encode the overlap between the instinct to approach a reward and the instinct to avoid a new (anxiogenic) space, as the under-activation only occurs when both components are present. We also found that the ICa-LH neuronal activity was not increased in anxiogenic spaces, in the classical anxiety tests, suggesting that these neurons are not relevant for the general role of the ICa in anxiety. We did not observe changes in activity in the negative valence tests. Since few data was collected, it is not sufficient to claim that these neurons do not have a role in negative valence encoding in general. To strengthen our results, we would need to perform more experiments using negative valence stimuli. In order to verify if the ICa-LH neurons are causally involved in the internal conflict between the instinct to approach a positive valence stimulus and the instinct to avoid a new space, we would need to perform optogenetic experiments. We expect that inhibition of this population's activity induces an increase in reward-seeking behavior.

We propose that the ICa-LH neurons are involved in the recognition of positive valence stimuli, in conjugation with anxiogenic stimuli. This behavioral context could be a potential target to study in physiopathology conditions. We can postulate that the ICa-LH neurons modulate how the positive valence stimuli are perceived in anxiogenic spaces, or how anxiety levels are modulated by the presence of positive valence stimuli.

As anxiety and valence play an important role in social interactions, we also studied the ICa-LH population during social observation. Mirror neurons are cells that fire when an individual performs a certain action, and when this individual sees another one performing the same action. These cells have been identified in humans and monkeys, in several brain regions, including the anterior insula in social contexts. However, the existence of mirror-like activity in the rodents' brain is still under debate. To address this question, we applied a social observation behavior protocol and performed preliminary recordings. Our hypothesis was that the ICa-LH neurons would show mirror-like activity, but our preliminary results indicated otherwise. However, to be certain that this population does not have mirror-like activity, the experiment should be replicated, ideally with a single-cell recording method.

This study defines the ICa-LH neurons as a possible neuronal substrate for encoding positive valence stimuli in anxiogenic contexts, and excludes the possibility that such neuronal population is contributing to the general hyper-activation of the anterior insula during high-anxiety situations.

Keywords: Insular cortex, Anxiety, Valence, Social observation, Fiber photometry.

INTRODUCTION

Anxiety and anxiety disorders

Definition

Fear is a natural emotional response to an immediate threat[1]. Anxiety is the anticipation of a future threat, with an uncertain probability of occurrence[2,3]. Both anxiety and fear responses are evolutionary relevant, since they allow the organisms to respond to or prevent exposure to harmful situations. However, fear consists of necessary surges of autonomic arousal leading to escape behaviors, or thoughts of immediate danger[2], while anxiety is associated with muscle tension, vigilance and avoidance behaviors[2].

Anxiety becomes pathological when it is sustained and / or provoked by innocuous stimuli[2,4]. An anxiety disorder must be diagnosed by a clinician, when it is persistent and lasts for 6 months, or more[2]. Panic attacks are one of the most common features of anxiety disorders, but can also be present in other psychiatric conditions[2]. The main symptoms are fear, avoidance behavior, and cognitive ideation[2]. There are different types of anxiety disorders, depending on the objects or situations that trigger the symptoms[2]. The main anxiety disorders listed in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) are separation anxiety disorder, selective mutism, specific phobia, social anxiety disorder (SAD), panic disorder, agoraphobia, generalized anxiety disorder (GAD), and substance / medication-induced anxiety disorder[2].

The American psychologist Charles Donald Spielberger introduced the concepts of state and trait anxiety, and developed the state-trait anxiety inventory[5]. Trait anxiety is defined as the individual's predisposition to respond, and it is seen as a personal feature, that is not dependent of the moment[6]. State anxiety, on the contrary, is anxiety about an event - the transitory emotional state of physiological arousal and apprehension, in the presence of an anxiogenic stimulus[6]. The difference between state and trait anxiety is analogous to potential versus kinetic energy[7]. State and trait anxiety do not mutually exclude each other, they can co-exist in many contexts[4]. These are psychological metrics to distinguish normal from pathological anxiety, but do not consider the neuronal mechanism behind the phenomena[4].

Animal models of anxiety

Although the clinical definition of anxiety and its subtypes is relatively clear, there is still a lack of comprehension about the neuronal mechanisms that underlie this pathology. It is crucial to understand which circuits are affected, and how, in order to establish better therapeutic targets, at pharmacological and behavioral levels. Human studies are important to establish correlations, and pinpoint important brain structures involved. Animal studies, however, are essential to understand the causality between brain dysfunction and behavior.

Animal models of human pathologies are very useful to study its mechanisms and action of drugs, in a way that is not possible with studying only human subjects. The first animal models of anxiety were developed in rats, and have then been adapted to mice,

which are easier to use, and can be genetically manipulated more easily[8]. These models need to fulfill three validity criteria: first, the models needs to have behavior and biological changes which are observed in human patients (face validity), second the models' etiology must mimic the etiology of the disease (construct validity), and third, responses to pharmacological treatment need to be similar (predictive validity)[9]. Nevertheless, it is important to highlight that animal models of anxiety disorders do not aim to replicate all features of the disease, but to induce a state of anxiety that can be related to the pathological condition it is mimicking[10].

There are two main classes of anxiety animal models: unconditioned / ethological behavioral-based models, and conditioned models[8]. Ethological models are based on unlearned fear behavior[8], and are the ones used in this project. Conditioned models rely on learned responses or classical conditioning[8]. Examples of the different types of animal models of anxiety are illustrated in Figure 1.

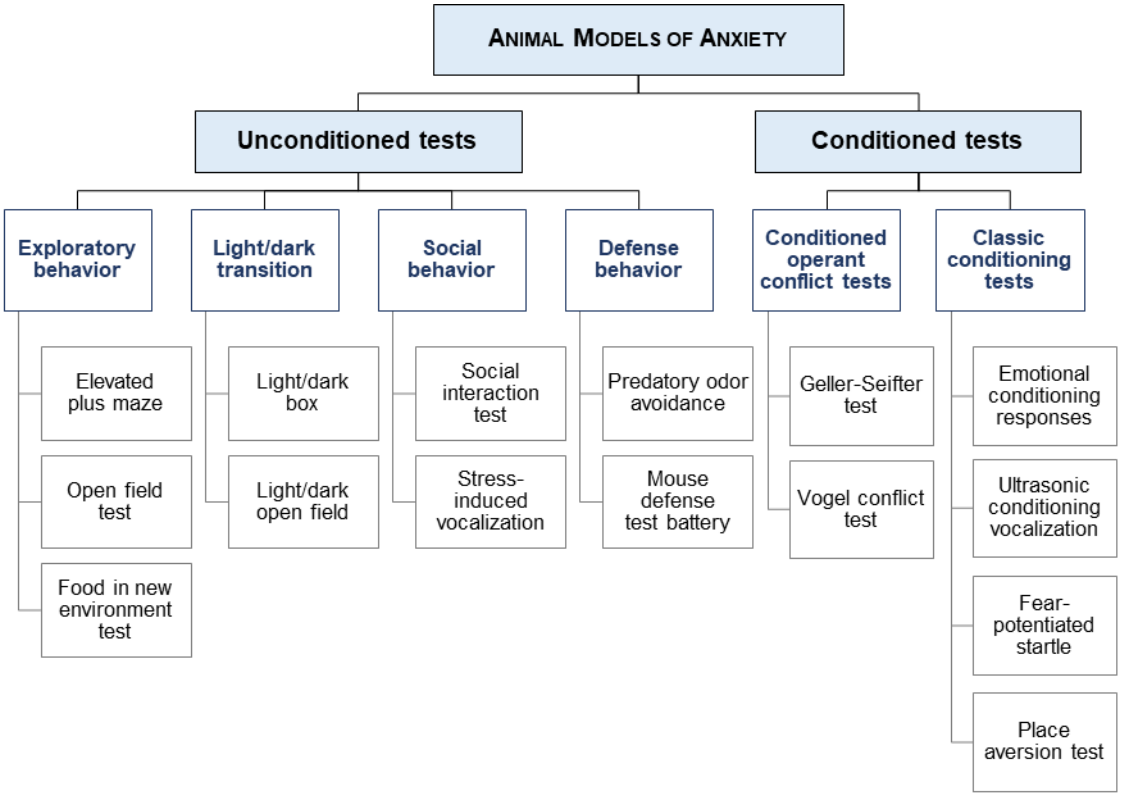


Figure 1: Anxiety models used in mice are based on two main groups of behavioral tests. (Adapted from Steimer, 2011 and Campos, 2013)

The unconditioned models of anxiety rely on behavioral tests[8] that allow us to study the unconditioned response to a new environment, that creates an approach-avoidance conflict in the animal[9]. In this project, we applied the elevated plus maze (EPM), the open field test (OFT), and the food in new environment test.

The elevated plus maze is a classical anxiety behavioral test, that was first validated for rats[11], and then for mice[12], in 1987. The animal is put in an elevated arena, in a plus shape, consisting of one open arm and one closed arm. Since it is elevated and exposed, the open arm is an anxiogenic space for the animals. The closed arm has walls that make it protective, turning it into a safe space (Figure 2A). The animals are conflicted between the natural drive to explore a new environment (the open arm) and protect themselves from potential threats (in the closed arm)[12]. In normal conditions, mice spend around 30% of the time in the open arm, and 70% in the closed arm[12]. Administration of anxiogenic drugs leads to an increase of the anxiety level of the animals, that tend to spend more time in the closed arm[11].

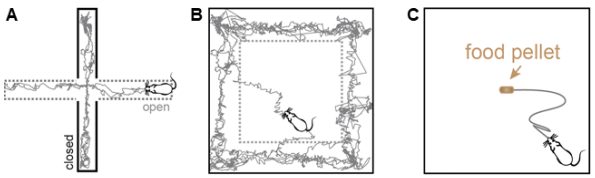


Figure 2: Classical unconditioned anxiety tests used in this project. A: Elevated plus maze (EPM). **B:** Open field test (EPM). **C:** Food in new environment test.

The open field test is another classical anxiety assessment test. Its use was first reported in 1934[13], and has since suffered some modifications, until the version that we use nowadays, with mice. In this test, the arena is squared, and the anxiogenic space is the center, while the safe spaces are the borders the field (Figure 2B).

The food in new environment test (also called as novelty-suppressed feeding test) was first developed for rats, to study the effects of antidepressant treatment in rodent models of anxiety[14]. Like the OFT, it relies on rodents' innate fear of novel spaces, but with an additional element of motivation. Since the animals are food-deprived before the test, the drive to eat conflicts with its fear of novel open spaces[15]. For the animal to eat, it needs to go into the anxiogenic area (Figure 2C). This tests relies on hyponeophagia: the tendency to avoid eating in a novel environment, and is directly related to anxiety[16]. The animal must choose between going into an anxiogenic space to get the reward, or to stay protected, but not get it. We also performed this test with sucrose and water, instead of a food pellet.

Animal models of anxiety with conditioned procedures can use operant conflict tests, or classical (Pavlovian) conditioning tests, like fear conditioning[8]. Operant conflict tests rely on natural responses to an environmental change, known as reinforcement, that can be positive or negative[9]. An example of positive reinforcement is when progressive lever-pressing leads to a greater food reward[9]. Negative reinforcement, for example, happens when the animal behaves in a certain way to avoid an unpleasant stimulus[9].

A classical fear conditioning paradigm involves the pairing of a neutral conditioned stimulus (for example, a sound) with an aversive unconditioned stimulus (for example, an electric shock)[17]. This learned association can be “unlearned”, or repressed[17] and when it is not, it can lead to anxiety disorders like agoraphobia or panic disorder[9]. The critical stage in the appearance of the phobia is the extinction phase, when the neutral stimulus is presented alone, without the aversive one[10]. Some individuals fail to repress the fear memory, and panic can arise[10]. This can be replicated in animals, using several experimental paradigms, as a model for anxiety disorders[18].

Biological link between anxiety and valence

Emotional valence is the subjective value assigned to sensory stimuli (objects, events, or situations)[19,20]. This term can be used to categorize emotions, for example, anger and disgust have negative valence, while happiness has positive valence[19]. It is crucial for the survival and adaptation of many species to discriminate external stimuli of negative and positive valence[21], thus the neural circuits encoding this capacity are conserved in the mammalian brain[22]. In normal conditions, stimuli of negative valence lead to avoidance and defensive behaviors, while stimuli of positive valence lead to approach and consummatory behaviors[20].

Valence assessment is disrupted in patients with anxiety-disorders[23]. Indeed, anxiety disorders patients and non-clinical high trait anxiety individuals both present an attentional bias for stimuli of negative valence[24,25]. For example, anxiety increases negative interpretations of ambiguous sentences and scenarios[20,26]. Additionally, high trait anxious individuals are more strongly engaged with a threatening stimuli and have higher tendency to avoid it, after its presentation[27]. It is widely established that people who are more vulnerable to anxiety tend to have an attentional bias towards threatening stimuli[24]. This is different than the natural "valence bias" in neuronal populations, that consists on the proportion of cells encoding positive of negative valence among the whole population[21].

It is important to note that this bias is probabilistic, and there is a high variability within groups and individuals. That is, the bias is not stable across time, task, stimuli, and setting[24]. Besides, the reliability of the current assessment tasks has been questioned, and can be part of the cause for the internal variability[24]. Nevertheless, it is strongly accepted that the neural circuits encoding emotional valence and anxiety overlap as there is a correlation between negative valence bias and the anxiety level[4]. The insular cortex is a key structure in this regard, since it is involved in anxiety disorders and valence processing, among other functions, like regulating interoception[28].

Empathy

Operational definition

Empathy is the ability to predict, or project oneself into the responses of others, whether individuals or groups, particularly attitudinal socio-emotional responses[29]. It is believed to be one of the skills that have supported our species' evolutionary success, and it is seen as evolving in layers (Figure 3), depending on emotional and cognitive processes, and leading to different behavioral manifestations[30,31].

Neural resonance is the tendency for neural systems to overlap between a perceiver (observing) and a target (experiencing the emotional state), and it is tied to experience sharing[30]. Mentalizing ("explicitly considering targets states and their sources") involves cortical temporal structures related with self-projection[30]. It was suggested that there is a coactivation of the neural systems involved in experience sharing and mentalizing, during complex social tasks[30].

Empathy is an evolutionary process, and it can be seen as beneficial or maladaptive. For example, by witnessing a negative emotion in others, the observer can better avoid potential risks, such as predation or poisoning[32], which is positive. However, the observer can also avoid responding to the pain of others and keep its distance, not helping the conspecific[31]. In such cases, there isn't activation of shared representations[31].

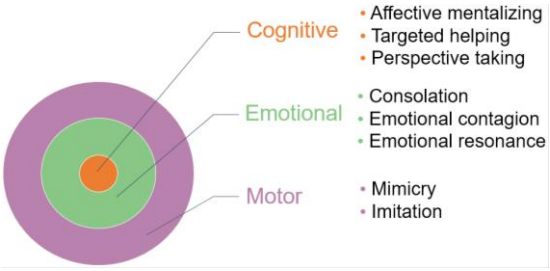


Figure 3: Model of the evolution of empathy. The most basic component is motor mimicry (or experience sharing) and emotional contagion, then comes empathic concern and consolation (altruistic helping), and the most evolved is perspective-taking (or mentalizing) and targeted helping. These paths all lead to the same goal, but there are different neural systems involved with each one. (Adapted from De Waal & Preston, 2017)

Usually, the affective and cognitive components of empathy are considered as separate, but they can be shared in bottom-up and top-down forms of empathy[31]. For example, when the observer directly perceives the emotional state of the target, which activated the personal representations in the observer's brain, or when the observer doesn't perceive the target, but remembers it, using brain areas related with memory, that then connect with emotion regulation areas[31]. In this case, the stimulation comes from inside the mind and not from the external world, but it also activates affective regions.[31]

Can rodents have empathy?

The existence of empathy in lower animals is a topic of research which has not been touched for more than a century, for being considered taboo[33]. It was thought that only human beings are capable of having higher cognitive and emotional skills like empathy, sympathy, or altruism[33]. However, this is a valid scientific question that can help us understand evolution and lead us to the development of animal models. One argument against the existence of empathy in rodents, is that empathy requires emotional contagion, which is a higher cognitive process, that rodents might not have[34].

Some studies have focused on the altruistic aspect of empathy, and showed that rodents and primates help their conspecifics when in distress and deny themselves food in order to avoid their conspecific to receive electrical shocks[31]. This type of behavior is greater if the conspecific receiving the shock is a familiar or a cagemate - showing that that avoidance of aversive stimuli cannot be the only reason for altruistic behavior[31] and that familiarity plays a role in empathic behavior[35].

Most of the work on empathy in mice is focused on fear learning. It was demonstrated that mice freeze when they see a conspecific being subjected to a footshock[36], indicating that the animals recognize fear responses in conspecifics. Observational fear learning is conceptually and empirically similar to affective empathy, so it is possible that the neuronal circuits underlying both processes overlap and / or communicate with eachother[32].

This model for empathic pain is widely used and it is largely accepted that it is based on a medial form of empathy (emotional contagion)[33]. Another study, using formalin as a nociceptor, showed that licking behavior was increased in mice that were injected with a lower dose but that were in contact with cagemates that received a higher dose, and vice versa[37]. Together with the fear learning studies, this work shows that mice are able to experience emotional contagion, which is one step towards feeling empathy[37].

Empathy for pain has also been observed in humans, where there is activation of the insula and other important regions for the affective component of pain[33]. A meta-analysis confirmed the relevance of the insular and the anterior cingulate cortices for empathic pain[33].

Mirror neurons

Definition

In 1992, scientists from the University of Parma developed an experiment to study how neurons from the rostral part of the inferior premotor cortex (area F5) of the macaque monkey's brain are activated in different behavioral situations[38]. Area F5 is homologous with Broca's area of the human brain[39]. This region is associated with mouth and hand movements, important for language acquisition, and the corresponding neurons have sensory and motor properties[40,41].

Incidentally, the researchers found that some F5 neurons are activated when the animal performs goal-directed hand movements, and when it observes the same movements being performed by the experimenters[38]. Later, in 1996, the same group published a new paper, where they called this cell population "mirror neurons" - MNs[40].

In Figure 4, the neuronal activity of a mirror neuron is represented[38]. This neuron was activated during precision grip, but not during whole hand prehension[38]. It fires both when the animal sees the experimenter grasping the food (Figure 4A) and when it is the animal grasping it (Figure 4B)[38].

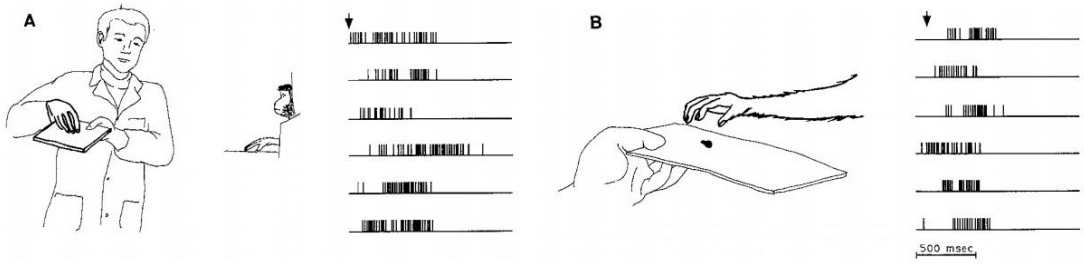


Figure 4. Mirror neurons in area F5 fire when the monkey observes an action being performed by the experimenter, and when the monkey performs the same action. Recordings of a mirror neuron, that selectively discharges during monkey grasping movements and during monkey observation of grasping movements made by the experimenter. **A**: The experimenter grasps the food. **B**: The monkey grasps the food. Arrows indicate the onset of grasping. (From di Pellegrino et al, 1992)

There are mirror neurons which are activated by observation vs performance of a certain action, and others that are inhibited[38]. Control experiments were performed to show that the firing of these neurons was specific to the hand food-grasping movements, and not due to movements of the animal that could not be noticed by the researchers[38].

Mirror neurons properties

Most of the MNs are selective for one kind of action. For example, a mirror neuron can fire in response to food grasping but not to a simple hand movement[40]. Control measurements were made in the dark, to be sure that the neuron activation was not due to the animal's vision of his own hand[40]. Mirror neurons can also have various congruence degrees[40]. The more specific the neuron is, in its motor and visual properties, the higher its congruency[40]. The high congruency of some MNs shows that their mirrorness cannot be because of unspecific factors of monkey-experimenter interactions, like food expectancy or motor preparation[40]. However, only one third of the MNs are strictly congruent[40].

Recent studies have reported the existence of other types of mirror neurons. Some can code auditory information[42], others are specifically activated when the monkey observes the experimenter using a tool to grasp an object[43]. Some MNs respond to facial expressions, ingestive, and communicative actions, raising the hypothesis that these cells are involved in understanding the emotional state of others[44]. One study showed that area F5 contains specific mirror neurons for communicative and ingestive mouth movements[45].

Mirror neurons in the human brain

The initial studies in monkeys produced a huge excitement in the scientific community, and soon researchers started to look for mirror neuron activity in the human brain[46,47]. One of the first studies shows that the motor and frontal cortices are activated during observation and execution of finger movements[46,47]. This raised the hypothesis that movement observation and execution share the same cortical network, based on a MN system[46,47]. Several studies in the anterior insular cortex (highlighted in the previous section) have led researchers to widely accept the existence of mirror neurons in this brain region, and in others, not specified here.

Most of the evidence for such conclusion comes from functional neuroimaging studies, that show an activation overlap between observation and action conditions[48]. Some authors believe that such studies oversimplify what MN activity is, since they define it only as a "strict congruency of observed and executed actions", not considering the fact that some MNs are only broadly congruent[49].

Only one study reported the existence of human MNs through direct measurements by single-cell recording techniques[50]. Patients with pharmacological intractable epilepsy were implanted with intracranial depth electrodes, to identify potential surgical targets. MNs were found in the medial frontal and temporal cortices, supplementary motor area, and hippocampus, using multi-unit and single-unit extracellular recordings[50].

Mirror neurons functions

The researchers that found mirror neurons proposed that they are responsible for the internal representation / recognition of an action and differentiation from other actions[40]. According to this view, the meaning of the observed action comes from the matching with the motor activity (and not from the emotions that it evokes)[40].

The original proposal for the MNs' function is now considered outdated. New suggestions have arisen from recent data: action understanding[51], imitation[44], empathy[44], emotion recognition[52], intention-reading[53], language acquisition and processing[54], etc. It has also been suggested that its dysfunction can be involved in disorders like schizophrenia[55], autism[56], and others[57].

Some authors believe that the adaptative and evolutionary advantage of having mirror neurons is to solve the "problem of other minds": their properties provide a quick and automatic mechanism of mirroring what is going on the brain of others, allowing us to understand their actions[44]. This led to the theories that defend that MNs are involved in empathy, emotional contagion and recognition[44]. Other authors question the importance of MNs in empathic processes[48], since some studies show that tasks where social judgment and empathy are needed do not engage any of the brain regions where the mirror system has been identified[48].

In a recent meta-analysis, from human 52 studies, it was concluded that MNs are likely related to the cognitive component of empathy, but there is weak evidence for an association with the emotional and motor components of empathy[58]. This result gives no clear answer about the issue, but indicates where future research might go.

Mirror neurons origin

Although there is no solid evidence about this topic[59], there are two theories aiming to explain the origin of mirror neurons: the associative account, and the genetic account[57]. The genetic account argues that MNs have arisen from natural evolution, and selection pressure favored the function of action understanding, which means the origin and function are correlated[49,60]. The associative account suggests that it is experience that leads certain cells to acquire "mirrorness"[49,60]. Hence, MNs might be important for action understanding and cognition, but not necessarily[49,57,60]. MNs are present, not only in cortical areas, but also in more ancient structures, like the basal ganglia, suggesting that the mirroring mechanisms might be evolutionary older than those originally discovered[59].

Studying mirror neurons in rodent models

Non-invasive techniques, which have been used in humans to study MNs, only allow us to see activity overlap between different contexts. With animal models, we can document field properties and understand how they are related to behavior. Mice can be genetically modified, we can apply pharmacological approaches and measure and manipulate neuronal activity at a cellular resolution. This is especially important, since MNs may not be in a high

quantity (in the original experiments, they made up less than 17% of all recorded cells[61]). Besides, rodent models are easier and cheaper to maintain than monkeys[33,57].

The existence of MNs in the rodent's brain is a question in debate. If we accept the associative account, we can hypothesize that rodents have a mirror system[62], but not all authors welcome this idea. Some authors claim that rodents are not a suitable animal model to study human MNs because of the difference in cortical surface area between the two species. However, recent data has demonstrated the existence of MN activity in the anterior cingulate cortex of rats[63]. Additionally, mirror-like cells encoding spatial information of a conspecific have been identified in bats'[64] and rats'[65] hippocampus, although this finding could not be reproduced by other groups[66].

Studying mirror neurons in the context of empathy in rodents is important to develop and validate better animal models of empathy. These models can play a role in a better understanding of empathy, in of bio-psychosocial-behavioral paradigms, thus helping bridging the gap between different fields of study[33].

The insular cortex

Johann Christian Reil, a German physician who lived in the 19th century, was the first to name and describe the insular cortex (IC or insula), also named insula or island of Reil[67]. This discovery was immortalized in Henry Gray's anatomical work[67]. In primates, the insula "lies folded deep within the lateral sulcus of each hemisphere, hidden below parts of the frontal, parietal and temporal lobes, which form the opercula" and its connectivity varies along the anterior-posterior (AP) axis[68]. In mice and in rats, the insular cortex "lies exposed on the lateral surface of the hemisphere, mostly along the rhinal fissure"[68]. There is high homology between the human and the rodent insular cortex (Figure 5), which makes the use of animal models, together with circuit mapping techniques, an important tool to understand its connectivity and functionality[69].

The insular cortex activity is modulated by a variety of functions: sensory processing, representing feelings and emotions[68] (for example, mediating fear[70] and anxiety[71,72]), controlling emotional valence[73], autonomical and motor control[74], risk prediction[75], decision-making[76], bodily- and self-awareness[77,78], and empathy[79,80]. The insula is also affected in many brain disorders including anxiety[81], depression[82], addiction[83], autism[84] and schizophrenia[85]. The IC transcriptome, recently characterized, is in line with its functions, since there is "enriched expression of genes associated with mood disorders, learning, cardiac muscle contraction, oxygen transport, as well as glutamate and dopamine signaling"[86].

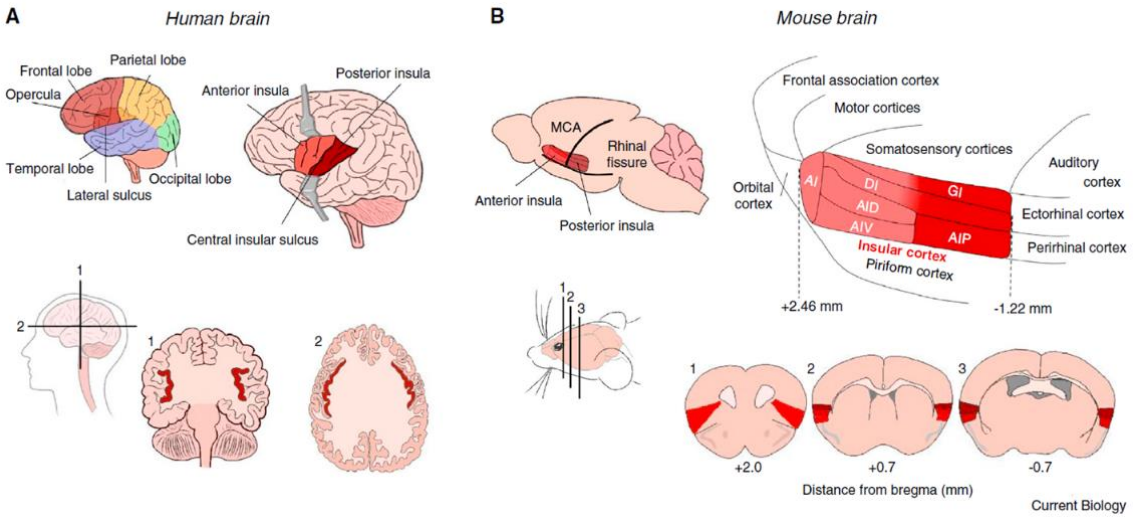


Figure 5. The human (A) and mouse (B) insular cortex. Top: Brain localization, subdivisions, neighboring cortical regions. **Bottom**: Coronal cross sections at different levels reveal the location and layers of the mouse insular cortex. MCA: medial cerebral artery. AI: agranular insular cortex. AID: agranular insular cortex, dorsal part. AIV: agranular insular cortex, ventral part. AIP: agranular insular cortex, posterior part. DI: dysgranular insular cortex. GI: granular insular cortex. (from Gogolla, 2017)

General connectivity pattern of the insular cortex projections

The insula is integrated in an extensive and complex network, which has been studied since the 1980's decade[87]. Initial studies used retrograde and anterograde tracers to study the insular cortex' connections throughout the whole brain, in rats[87]. The IC receives information from the external (sensorial) and internal (interoceptive) environments of the body, from direct thalamic and horizontal cortical afferents[87,88]. Many of these inputs project to different insular sensory regions, but none of such regions processes only its major sensory input: the whole IC receives cross-modal afferents, which makes it a multimodal integration site[68,87].

The insula is also reciprocally connected with the limbic system[89]. The amygdala, a complex structure involved in emotional regulation processes[22], including fear learning[90], projects to the IC, which then send projections to several amygdala nuclei[91,92]. The monosynaptic excitatory projection from the IC to the central amygdala (CeA) is necessary for the suitable response to taste-predicting cues[93] and it was identified as a potential target for relapse prevention[92]. The anterior insula (ICa), in particular, has reciprocal connections with the basolateral amygdala (BLA)[94]. Additionally, there are reciprocal projections between the insular cortex and the anterior cingulate, orbitofrontal, and medial prefrontal cortices, that are important for cognitive, emotional and executive functions[68]. Projections from the IC to the nucleus accumbens and the caudate putamen are involved in motivation and reward[68].

Recently, Gehrlach et al. studied the connectivity of the whole mouse insular cortex, across the AP axis[95]. As it has been suggested in previous studies, the posterior and the anterior IC have different connectivity patterns, which suggests different functionality[95]. It is

shown that the insula is highly connected with other cortical regions, such as the sensory cortex, and the ICa is particularly connected with the prefrontal cortex[95]. Regarding subcortical regions, the IC sends the majority of its projections to the striatum, and receives most of its inputs from the amygdala and thalamus[95].

The insular cortex to lateral hypothalamus projection

The hypothalamus is a highly conserved region of the mammalian brain, and it has a critical role in keeping homeostasis[96]. To do so, it regulates many neuro-endocrine functions, it sends out signals that control and alleviate feelings of thirst, hunger, and fatigue, and it regulates body temperature, and circadian cycles[90,97].

The lateral hypothalamus (LH), in specific, is very important for controlling predation and evasion behavior[98], responding to discrete threat stimuli[99,100], regulating the sleep cycle[101], feeding and drinking behaviors, and reinforcement and motivational processes associated[102]. Electrical stimulation of the LH produces an augmentation in feeding behavior and a reinforced lever-pressing to get additional stimulation[102].

The LH neuronal population is heterogeneous: we can find excitatory glutamatergic and inhibitory GABAergic neurons[101]. In parallel, the LH has neurons producing different neuropeptides. The two most common are the hypocretin / orexin (OX) – producing neurons[103] and the melanin-concentrating hormone – producing neurons[104], which are spatially intermixed. OX neurons are especially relevant for feeding control in mice,[105] and are also activated to evoke defense responses to fearful stimuli[106]. There are two subtypes of orexin peptides (OX_A and OX_B) as well as two OX receptors (OX_1 and OX_2)[97], which have opposite roles in anxiety-related behaviors[107].

Early studies in rats have shown that the IC sends glutamatergic projections to the LH[89,108], that mainly rely on NMDA receptors[109], and are responsible to control food and water consumption, and cardiac function[110,111]. Microinjection of D,L-homocysteic acid, an excitatory aminoacid, in the insula elicits changes in arterial pressure and heartbeat, depending on the sub region of the injection, and the projection to the LH is involved in both effects[111]. Microsimulation of the rat insular cortex promotes tachycardia, and such effect can be abolished by injection of a synaptic blocker or a NMDA receptor antagonist in the LH[110]. Furthermore, there may be neuromodulation by opiates and neuropeptide Y of this neuronal projection[110].

A recent study focused on the anterior insula to lateral hypothalamus (ICa-LH) projection neurons in the context of feeding behavior[112]. An anterograde viral vector encoding for enhanced green fluorescent protein (eGFP) in a Cre-dependent manner was injected in the ICa of CaMKIIα-Cre mice[112]. Green fluorescence in the LH (Figure 6A) reveals the presence of axonal terminals, confirming the existence of an ICa-LH projection[112]. To confirm that it is an excitatory connection, the authors performed *ex vivo* recordings[112]. Holding the membrane potential at -70 mV and stimulating the LH terminals with 473 nm light induces EPSC (Figure 6B), which is blocked by the addition of the AMPA receptor

antagonist CNQX[113] (Figure 6C). Finally, a rabies virus-based retrograde tracing experiment confirmed that the ICa-LH projection is monosynaptic[112]

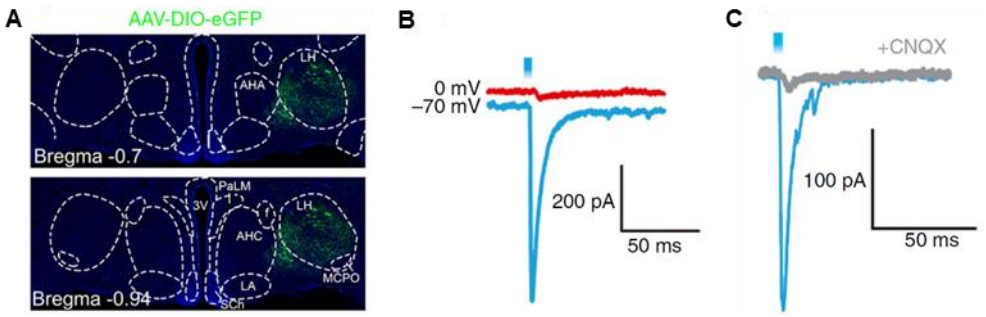


Figure 6. The anterior insular cortex (ICa) sends glutamatergic projections to the lateral hypothalamus (LH). A: Representative immunohistochemistry of images from the LH showing CaMKII^{+} fiber terminals in green after injecting hSyn-DIO-eGFP into the ICa of CaMKIIa-Cre mice. Scale bar, 250 μm. **B**: Light can evoke EPSCs (held at −70 mV), but not IPSCs (held at 0 mV). **C**: Single light evoked EPSC, which can be blocked by CNQX. (From Wu et al, 2020)

Insular cortex in emotion

Emotions are neural responses triggered by external and internal stimuli, which will trigger behavioral responses[20,118]. For example, the presence of a dangerous stimulus triggers an emotional response of fear[114].

The specific role of the insula in generating emotions is under debate, since it was shown that children born without cerebral cortex[117] and patients with bilateral insular damage still have the ability to experience somatosensory and interceptive stimulations and emotions[88]. This suggests that the role of the IC in emotions may instead be related with its cognitive functions, as the insula is highly connected with other cortical regions involved in memory, language, and reasoning[114].

Nevertheless, it is generally accepted that the insular cortex is important in the processing of the stimuli received from the internal milieu and external world[114]. There are two main pathways that guide interoceptive information from the body, until higher cortical regions (Figure 7)[114–116].

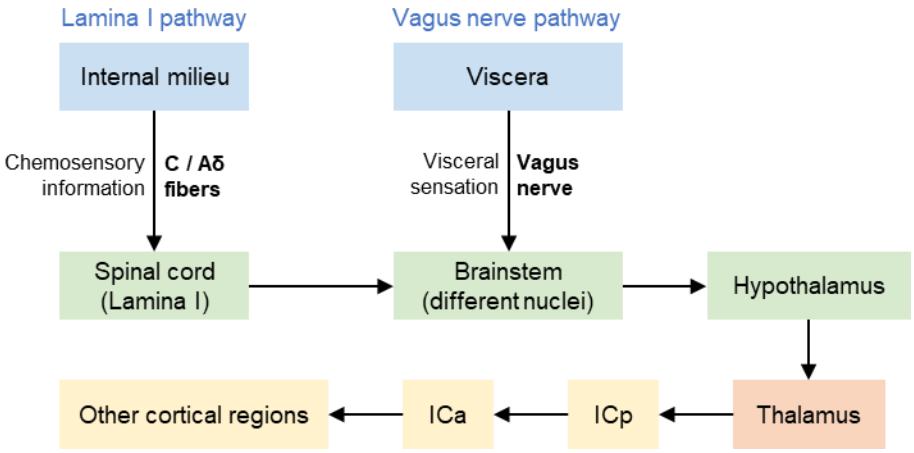


Figure 7. Interoceptive pathways and brain regions involved in sensing and mapping body states and emotions. Two main pathways convey information from the internal environment to the central nervous system. The lamina I pathway consists of C and Aδ fibers carrying information leading to several bodily responses, and converges in the lamina I, from where secondary neurons project to homeostatic centers in the brainstem. These centers project mostly to the ICp, via thalamus. The information collected is projected to the ICa, which connects with other cortical areas. The vagus nerve carries information from the viscera to the brainstem and hypothalamus. Each of these structures also projects directly to the IC. Crosstalk between both pathways allows the formation of integrated maps of body states. (Adapted from Damasio & Carvalho, 2013).

Interoception

One important function of the insula is to regulate interoception: the perception of internal bodily states[90].The IC receives input from other brain regions and sends outputs to maintain the homeostatic state of the body - a critical factor for emotion[119].

Several studies suggest that the anterior insula has a particularly important role in human awareness[77]. Mainly using fMRI, researchers have shown a diverse range of interoceptive stimuli that activate the ICa: thirst[120], taste processing[121], sexual arousal[122], itch[123], warmth[124], dyspnea[125] and bladder distension[126] are some examples. A particular PET scan study showed that the objective sensation of cool temperatures activates the posterior insula, while subjective rating of these stimuli correlates with activation of the anterior insula[127]. The authors suggested a posterior-to-anterior pattern of integration of the interoceptive information, in the insular cortex.

Besides subjective bodily feelings, the insula is also activated in association with emotions, like maternal and romantic love[128], anger[129], fear[70], and aversion[130], for example. Additionally, the ICa is active during the corresponding empathic feelings[77] (explained in more detail in the Empathy section). Moreover, the anterior insula is active in tasks involving cognitive choices, attention, time perception, and awareness of the visual image of the self[77]. It is the only brain region activated in all of these contexts, and all of them engage the awareness of the subjects. This raised the hypothesis that the ICa constitutes a neural correlate of awareness[77], which supports the theory that the insular cortex is an integration site between interoception and emotions (Figure 8)[68,69].

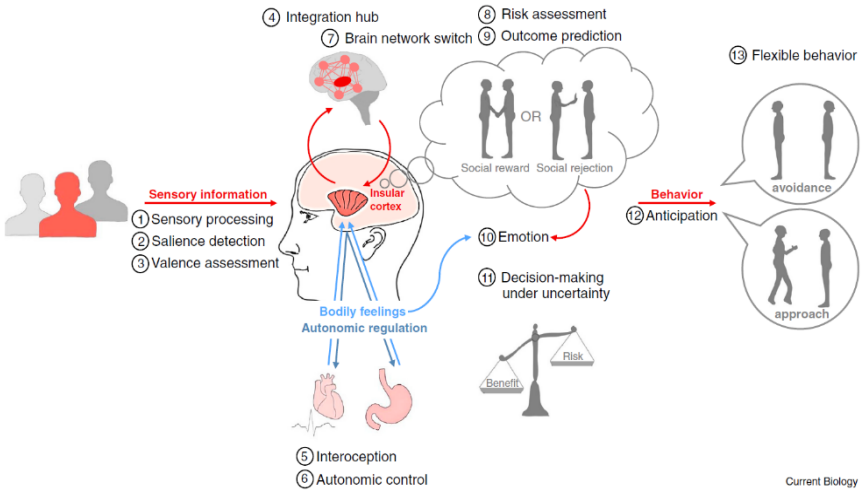


Figure 8. Integrated model of the insular cortex functions, in the context of a social encounter. The IC receives multisensory information from the outside world, processes the salience of those stimuli and evaluates its valence, by integrating information from multiple brain systems. It perceives bodily feelings and regulates the physical reactions caused by the situation. The interactions with other brain networks allow the assessment of the risk of the interaction by predicting the possible outcomes. Emotions can be caused by bodily feelings or cognitive processes. The insula is also important in select behavioral responses in face of each situation, anticipating the outcome of the decision and adapting the behavior accordingly. (From Gogolla, 2017)

Anxiety

The insular cortex is linked to anxiety in many ways. Insular activity is altered in anxiety patients[131], and anxiety-prone individuals have an increased insula activation during emotion processing (response to emotional faces)[132]. Additionally, the IC connectivity with the limbic system[91,92] makes it a key structure to study altered homeostatic physiological sensations - an important component of anxiety[28].

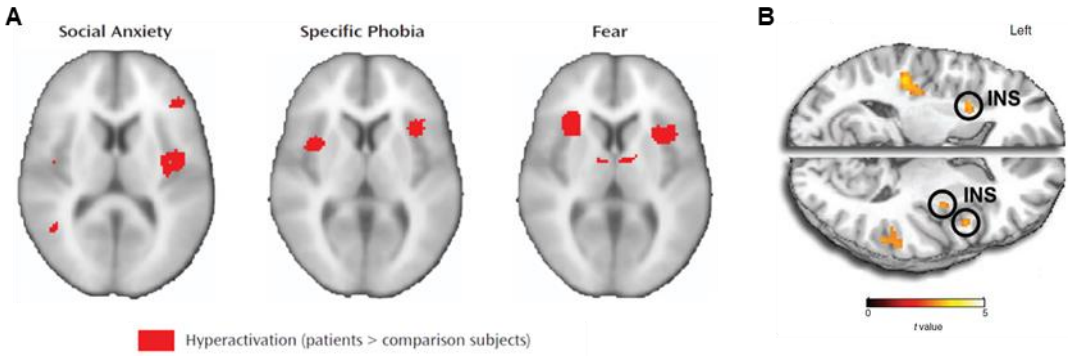


Figure 9. The insular cortex is hyper-activated in patients with social anxiety disorder and specific phobia. A: fMRI shows the insular cortex is hyper-activated in patients with social anxiety and specific phobia, in comparison with healthy subjects during fear conditioning. (From Etkin & Wager, 2007) **B**: fMRI shows the insular cortex is hyper-activated in patients with social anxiety in response to negative (> neutral) images, in comparison to healthy subjects. (From Phan et al. 2009)

The insular cortex is particularly relevant to understand anxiety disorders because it is the only brain region (together with the amygdala) that is hyper-activated in patients with social anxiety disorder (SAD)[81,133] and specific phobia[81] (Figure 9A). Furthermore, patients with SAD have an increased insular and amygdala activity when looking at images with negative valence (versus neutral), compared with healthy subjects[133] (Figure 9B). The IC is also activated in anticipation to emotionally aversive stimuli – a hallmark of anxiety[134]. The activity of the insula was increased when individuals selected a "risky" response, as opposed to a "safe" one, and the degree of activation is correlated with harm avoidance and neuroticism of the subject[134].

The insular cortex has been studied in the context of anxiety treatment, with citalopram[135]. This molecule is a serotonin uptake inhibitor – one of the most common types of anxiolytic drugs[136,137]. This type of treatment blocks serotonin reabsorption to the pre-synaptic cell, leading to the increase of its extracellular concentration in the brain[138]. It was demonstrated that the IC activity is reduced (weaker fMRI signal) after citalopram treatment of generalized anxiety disorder (GAD) patients, comparing to the pre-treatment condition[139]. The study was done by measuring the self-reported anxiety of the patients after listening to verbal descriptions of a personal worry or a neutral statement, before and after treatment[139]. This methodology is interesting, because it is a practical example of how the circuits encoding anxiety and valence are overlapping.

The data summarized so far in this Introduction come only from human studies, which always rely on non-invasive techniques, like fMRI or EEG. These techniques measure brain activity at a superficial level, but obviously do not allow for its manipulation. For that reason, all human studies are correlational. There are some techniques to stimulate neuronal activity in humans, however, they don't allow us to study specific neuronal populations, and there are not systematic studies using such techniques.

Only with animal models, including rodents, we can apply invasive methods in a systematic way, to help us establish a causal relationship between neuronal activity and behavior. With optogenetics, it is possible to activate or inhibit specific neuronal populations, and see the consequences on behavior, thus infer causality. Fiber photometry or *in vivo* electrophysiology are also useful to record neuronal activity, at a population or single cell level, respectively. However, there are still very few studies exploring the role of the insular cortex in anxiety-related behaviors and valence, at a circuit level. That knowledge is essential to understand the neuronal mechanisms behind the pathology, an in the long run, define therapeutic targets for it.

In 2019, a pharmacological study in rats applied different drugs in different sub-regions of the insula (along the dorso-ventral axis) to assess their role in anxiety-related behaviors[71]. AMPA receptor antagonist CNQX was used as an inhibitor and GABA receptor agonist Bicuculline was used as an activator of the insular neurons. The effects of several injections were measured, and it was concluded that the IC has a role in modulating anxiety depending on its dorso-ventral location (Figure 10A). Specifically, the ICa has anxiogenic effects (Figure 10B) while the ICp has anxiolytic effects[71].

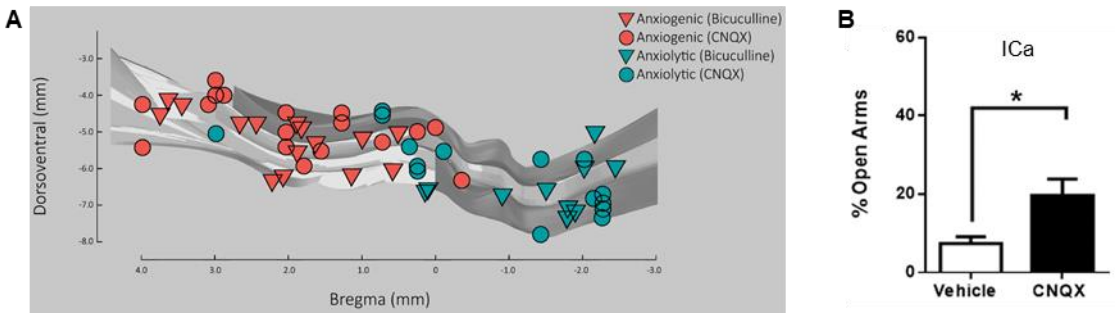


Figure 10. The insular cortex activity has different anxiety-related effects, along the dorso-ventral axis. **A**: Anxiolytic and anxiogenic roles along the insular cortex based on CNQX and Bicuculline microinfusions. Each triangle/circle represents an injection case (circles, CNQX; triangles, Bicuculline). The anxiolytic/anxiogenic classification was qualitative and defined as the role of that particular location in anxiety. The role in anxiety for each injection site was estimated based on the difference between the time in the open arms of the EPM on each case and the average of the respective control. Given that CNQX is an inhibitor, final values for CNQX were multiplied by –1. Final values <0 were considered anxiogenic and >0, anxiolytic. **B**: Percentage of time spent in the open arms of the EPM when the animals were injected with vehicle solution (white bar) or with the AMPAR antagonist CNQX (black bar) into the ICa. (From Méndez-Ruette et al, 2019).

The anxiolytic role of the ICp was corroborated in another study, using optogenetic inhibition in the EPM[140]. However, optogenetic activation did not lead to changes in the same test[140], which makes the role of ICp in anxiety unclear. Additionally, unpublished data from the Beyeler lab suggest the ICa is more relevant in anxiety-related behaviors than the ICp. Specifically, using fiber photometry recordings from all glutamatergic neurons of the two sub-regions, they found a significant increase of the global activity in the open arm of the EPM in the ICa, but no changes in the ICp.

It can be concluded that the insular cortex, in particular the anterior section, is a relevant structure for anxiety-related behaviors. However, more studies with rodents are necessary in order to establish a solid causality between the neurocircuitry and the behavior. In the long run, this knowledge can be used for drug development: anxiolytic treatments that target the specific brain regions, or neuronal populations, responsible for anxiety.

Valence

The role of the insula in valence has been addressed in recent studies. Optogenetic activation of the whole posterior IC is aversive to mice (Figure 11A), suggesting that its activity “transmits a negative valence signal, causing animals to exhibit defensive reactions”[140]. In a projection-specific experiment, it was demonstrated that the ICa-BLA projection is involved in sweet taste processing, and its optogenetic stimulation is appetitive for mice[73] (Figure 11B and C). On the contrary, activation of the ICp-CeA projection is aversive (Figure 11D and E), and this projection is involved in bitter taste processing[73].

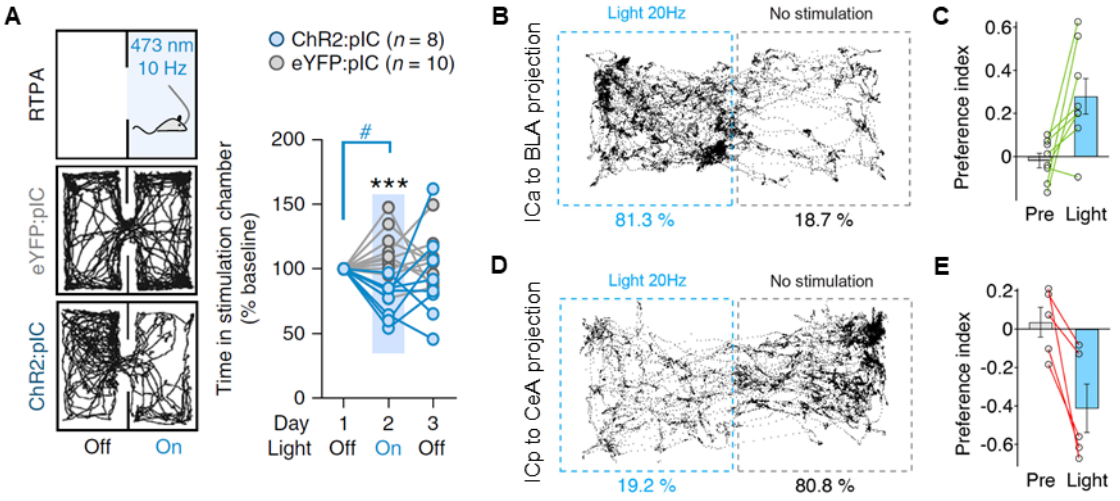


Figure 11. The insular cortex in valence. A: Optogenetic activation of the ICp produces RTPA. Left: Representation of the assay and representative locomotor traces of eYFP- and ChR2-expressing mice. Right: light activation of ICp neurons results in place aversion in ChR2-expressing mice (***p=0.0008 for ChR2-expressing compared to eYFP-expressing mice with the laser on; #p=0.0463 for ChR2-expressing mice with the laser off versus these mice with the laser on. (From Gehrlach et al, 2019). **B-E**: Optogenetic activation of different IC projections produces different valence-related behaviors. (Adapted from Wang et al, 2018). **B**: Optogenetic activation of the ICa-BLA projection produces RTPP, as represented by the locomotor traces. This animal spent 81.3% of the test time in the stimulation chamber. **C**: Quantification of preference index before and during light stimulation. (p=0.0156). **D**: Optogenetic ICp-CeA projection produces RTPA, as represented by the locomotor traces. This animal spent 80.8% of the test time in the no stimulation chamber. **E**: Quantification of preference index before and during light stimulation. (p=0.0207).

Empathy

Different brain areas are activated when empathy is triggered by visual cues or by abstract symbols: premotor cortices (important for action understanding), or prefrontal cortex (important for inference of mental states)[48]. However, the ICa is activated independently of the task, which suggests that its functions are related with different pathways[48].

The neuronal circuits responsible for the experience of pain are relatively well understood[141]. The network of cortical and subcortical structures that are responsive to pain experienced by oneself is called pain matrix[142], and it can be divided into the sensory-discriminative component and the motivational-affective component[48].

Intracerebral stimulation experiments show that the anterior insula (and the dorsal anterior cingulate cortex - ACC) is specifically activated during anticipation of pain[143], and it is not related to nociception[144]. On the contrary, posterior insula stimulation triggers painful sensations[145]. This corroborates the different functionality of the insula along its AP axis and suggests the ICp is more important for the actual sensory experience of pain, while the ICa is relevant for the affective dimensions of it, and it can have a role in empathy for pain[48].

A fMRI study in human couples found that only the anterior insula and the dorsal ACC are activated when one individual sees the partner being subjected to pain[146]. So, empathy for pain does not require the whole pain matrix, but only the components implicated in the affective dimension of the pain experience[146].

It can be suggested, then, that the ICa (and the ACC) have a dual function: they are important for the subjective representation of emotions, and constitute the neural substrate that allows us to understand the emotions of others[146]. A higher brain activity in these regions is correlated with higher scores on empathy tests, which is in line with this idea[146]. Other studies show that the IC is active both when we experience disgust and when we see others experiencing it[90], which further supports the theory that the IC is important for empathy.

Activation of the anterior insula during emotion sharing is not exclusive to pain. Observing other people experiencing disgust[79] or observing other people smiling[147] also elicits activation of the ICa. In rats, researchers demonstrated that optogenetic inactivation of the insula, or blocking of the insular oxytocin receptors abolishes social affective behaviors, during a social affective preference task[148].

Naturally, there are also many structures that are activated exclusively only during self-experiencing or during observation[48] – there is not a perfect overlap in the neuronal encoding of self- and other-related experiences. Nevertheless, shared networks are certainly a relevant mechanism to understand intersubjectivity, and the anterior insular cortex is an essential brain structure in that regard[48].

In conclusion, the insular cortex, since it is activated both during self-experience and observation of emotional states[32], is considered a central region for generating and predicting emotional states of the self and others (together with other brain regions, like the amygdala and the ACC[32]), which is the basis of affective empathy[48].

Mirror neurons in the insular cortex

Mirror neurons are present in the insula, in the human brain[33,149]. Insular MNs are activated both when the patient participates in an experience of pain or disgust and when they see it in others[33,149]. Furthermore, the anterior insula and the cingulate cortex are activated in humans, when pain is directly experienced through electrical stimulation and when the participants observe their partner receiving a painful stimulation[33,149].

Record and manipulate neural activity

Fiber photometry

To record neuronal activity in a projection-defined manner, fiber photometry relies on a genetically encoded calcium indicator – GCaMP - first developed in 2001[150]. It consists on a fusion between the green fluorescent protein (GFP), calmodulin (CaM), and the peptide M13, which is a fragment of myosin light chain kinase[150].

When Ca^{2+} is not present, GFP is bound to a chromophore, in a solvent-exposed neutral state[150–152]. Upon synaptic transmission, Ca^{2+} is released and bound to CaM, which promotes a change in the electrostatic potential around the chromophore. This leads to a conformational change in the interaction between CaM and M13[150–152]. Consequently,

solvent access is blocked and the deprotonation of the chromophore is restored, causing a spectroscopic transition, that is measured as an increase in fluorescence[150–152].

There are several versions of GCaMP. In this work, we used GCaMP6 (Figure 12). It was created via site-directed mutagenesis, in order to have a faster kinetics and improved affinity to the M13 peptide, leading to a better detection of the Ca^{2+} signals[153]. GCamP6 responds to single spikes with 100% probability and can be stably expressed in neurons for at least 4 weeks, not affecting the electrophysiological properties of the cells[153].

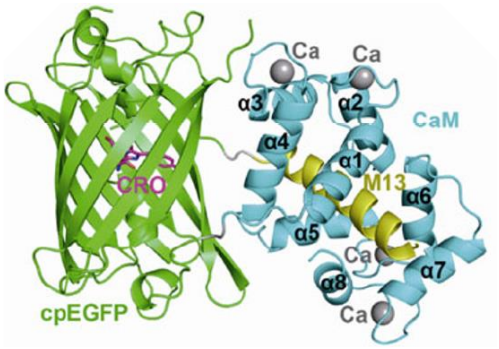


Figure 12. Crystal structure of GCaMP6. M13 peptide in yellow; cpEGFP in green; CaM in blue; chromophore (CRO) is shown as stick models in magenta; four Ca^{2+} bound to the CaM as grey spheres. (From Nakai et al, 2001)

Random mutagenesis was applied in GCaMP6 and newer versions were developed[154]. However, GCaMP6 has higher sensitivity and rapid kinetics, which are important for the detection subtle and fast neuronal activities[153]. After being tested *in vitro*, GCaMP6 validity was confirmed *in vivo*, in zebrafish, flies and in pyramidal neurons of the mouse visual cortex[154]. There are subtypes of GCaMP6, with different kinetics: GCaMP6s, GCaMP6m and GCaMP6f, with slow, medium, and fast kinetics, respectively[152]. We used GCaMP6m for a better compromise between signal intensity and sensibility.

Optogenetics

To have a technique that allows us to manipulate and record neuronal activity at a cellular level, in a temporally precise and non invasive manner, has been a long term goal in Neuroscience[155]. Optogenetic approaches, developed in the beginning of the XXIst century have come to fill that need. This technique uses light (*opto*) and genetically encoded proteins (*genetics*) to target specific neuronal populations[156]. The most used optogenetic probe is channelrhodopsin-2 (ChR2) - a rapidly gated light-sensitive cation channel that is naturally present in unicellular green alga *Chlamydomonas reinhardtii*[157]. When ChR2 is expressed in neurons, neuronal spikes can be elicited with blue-light pulses[157].

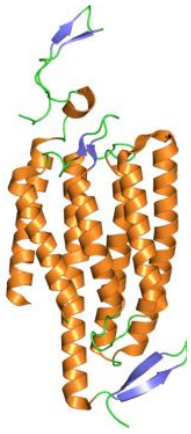


Figure 13: Crystal structure of ChR2 (from Kato et al, 2012).

Similarly to voltage-gated ion channels, channelrhodopsin (Figure 13) is expressed in the cell membrane, and when it is opened, it lets positive-charged ions, like Na^{+}, flow inside the cell where it is expressed[157]. To be opened, ChR2 needs to be stimulated with blue light, which promotes a conformational change[157].

Optogenetics can be used *in vitro*, paired with eletrophysiology techniques, for example: electric currents can be detected in neurons that receive input from upstream

neuronal populations that express ChR2[157]. The first *in vivo* application of optogenetics was made in *C. elegans*, where ChR2 was expressed in muscle cells, and induced strong contractions, that would normally be elicited by lechanical stimulation[158]. Optogenetics was first applied in freely moving mice, to evaluate the role of OX neurons in the hypothalamus in the transitions from the sleep to awake state[159]. Direct and selective activation of this neuronal population leads to an increase in the probability of transition to wakefulness[159].

Research objective and hypothesis

With this Introduction, we aimed to provide a brief review of the most relevant scientific discoveries in the context of this project. In the first section, we explained the basic concepts of anxiety, its biological link with emotional valence, briefly defined empathy, and explored mirror neurons' properties and potential functions.

Then, we focused on the insular cortex: a crucial brain region for the aforementioned functions. We highlighted how the anterior insular cortex is a crucial region in the circuit overlap between anxiety and valence, and characterized the ICa-LH projection, which is the focus of this project. We also mentioned that mirror neurons are present in the human insula, underlining the importance of understanding whether they exist in the rodent brain. Lastly, we briefly explained cutting-edge techniques to record and manipulate neural activity.

The first objective of this project was to contribute to the wide challenge of identifying neuronal circuits responsible for anxiety, which might be disrupted in anxiety disorders. In the long term, those circuits could become specific neuronal targets for therapeutic strategies. Specifically, we aimed to understand whether and how the ICa-LH neurons are involved in anxiety-related and in valence-related behaviors. Based on clinical imaging studies, our working hypothesis was that the ICa-LH neurons are hyper-activated in anxiogenic spaces.

In the second part of this project, we intended to address the possibility of mirror neurons existing in the rodent brain. As we can apply circuit mapping and recording techniques in rodents, that cannot be used in humans, rodent models can potentially provide a deeper knowledge on the properties of mirror neurons. We know that mirror neurons are present in the human insula in contexts where subjects feel empathy. We hypothesized that the ICa-LH neurons in the mouse brain would show mirror-like properties in a social observation context (a primary form of empathy).

Both objectives of this project aimed at a better understanding of the functionality of the ICa-LH neurons, which can be useful to understand complex behaviors related with anxiety, valence assessment, social observation, and even empathy.

MATERIALS AND METHODS

Experimental animals and housing conditions

All the experiments were performed with adult male and female C57Bl6/J mice, from Charles River Company. The animals were housed in controlled conditions of temperature and humidity environment, on a reversed light/dark cycle (light on at 8h to 20h) and with *ad libitum* access to food and water. Animal maintenance, treatments and experimental procedures were conducted according to French governmental regulations, and approved by the ethical committee and the Ministry of Education, Research and Innovation.

Stereotaxic surgeries

Animals were anesthetized with 5% isoflurane, then the concentration was decreased to 1.5% and kept during the whole procedure. Metacam, a pain-killer (NSAI: Non Steroidian Anti Inflammatory) was injected intraperitoneal at 5 mg/kg. During the whole surgical procedures, the animals' eyes were protected with Ocry-gel to maintain lubrification, and the body temperature was kept at physiological levels, using heat pads at 42°C. The animals' head was shaved and disinfected with betadine and ethanol 70%. Then, the head was fixed, and Bregma and Lambda positions adjusted to be at the same dorsoventral level. The anterior-posterior tilt between Bregma and Lambda and the medio-lateral tilt at +2.5 mm and -2.5 mm from Bregma were set to be at maximum 20 µm.

Viral injections were delivered using glass micropipettes (3–000-203-G/X, Drummond), made by a puller (PC-100, Narishige). The injection rates were controlled using a Nanoject III (3–000-207, Drummond). After performing a craniotomy above the target regions, the virus was loaded inside a micropipette that was introduced in the tissue at 10 µm per second speed, until 100 µm deeper than the injection DV coordinate, to create a pocket for virus diffusion. After moving the micropipette 100 µm up, 300-350 nL of virus (titer 6.362E+13 GC/mL) was injected at 5 nL per second. Then the micropipette was lifted up of 100 µm, left for 10 minutes, to allow for the virus diffusion, and removed at 10 µL per second.

Before the surgeries, the optic fibers stayed in ethanol 70% for at least 5 hours, and then were washed with sterile sodium chloride. The implantation was done 50 µm above the viral vector injection site, to record the activity of neurons cell bodies that project to the LH. After that, biocompatible cement and resin were used to keep it fixed in the brain, leaving the ferrule exposed, to be able to connect with the patchcord during behavioral assays.

At the end of the surgery, after letting the cement dry, the animals' heads were stitched and they were left recovering under a heat lamp, and monitored for the following 4 days. All the surgeries took between 2.5 and 3 hours.

Head fixation

For the social observational experiment, described in detail below, the mice underwent an extra step to cement a stainless steel headbar behind the optic fiber ferrule. The animals were anesthetized a second time, after all the anxiety and valence assays, and put in the stereotaxic frame as described before. Then, a small incision was made on the skin and the headbar was put on top of the cement fixing the optic fiber ferrule, aligning the center of the bar with the sagittal suture. The bar was fixed with biocompatible cement and resin. High vacuum grease was applied around the fiber ferrule, to avoid the cement from getting in contact with it. After the cement dried, the skin was re-stitched and the animals were left to recover and monitored during the following days.

Fiber photometry recordings

Optic fibers

The optic fibers implanted in the anterior insula of the mice's brain have 400 µm diameter. They were hand-made and only the ones with efficiency above 90% were used in the experiments. The fibers were inserted in a metal ferrule, with 430 µm inner diameter and 1.25 mm outer diameter. During the behavior tests, the optic fiber implanted in the brain is connected to the fiber patchcord of the photometry system via a small zirconia sleeve, with inner diameter 1.25 mm.

Viral vectors expression

The viral vectors need approximately 4 weeks in order to be fully expressed in the neurons. For this reason, the recordings are made 4 weeks after the surgeries. In Sup. Figure 1, we show an example of quality signal check recordings over the third week after viral delivery. It is visible that by the end of the third week, the signal quality is better than in the beginning.

In order to express GCamP6m selectively in the cell bodies of ICa neurons that project to the LH, we used a Cre-dependent approach. In the ICa, we injected an adeno associated virus 9 (AAV9), carrying the GCaMP6m gene, under the control of the human synapsin promoter (Syn), for exclusive neuronal expression, and in a double-floxed inverted open reading frame (DIO), to be expressed in a Cre-dependent manner (Figure 14B). Its full identification is AAV9-Syn-DIO-GCaMP6m-WPRE-SV40 and the injection coordinates were AP/ML/DV -0.7/1.0/-5.25 mm from Bregma. In the LH, we injected the viral vector CAV2-Cre, in the coordinates AP/ML/DV -0.7/1.0/-5.25 mm from Bregma.

The canine adenovirus vector CAV2 carries the Cre-recombinase (cre) gene. After being injected in the LH, it travels retrogradely to the upstream regions, where the protein is expressed by the transduced cells. When the vector reaches the ICa, the Cre-recombinase inverts the GCaMP6m gene sequence in the other viral construct, if present in the same cell, and the Ca^{2+} indicator can be expressed as well (Figure 14B).

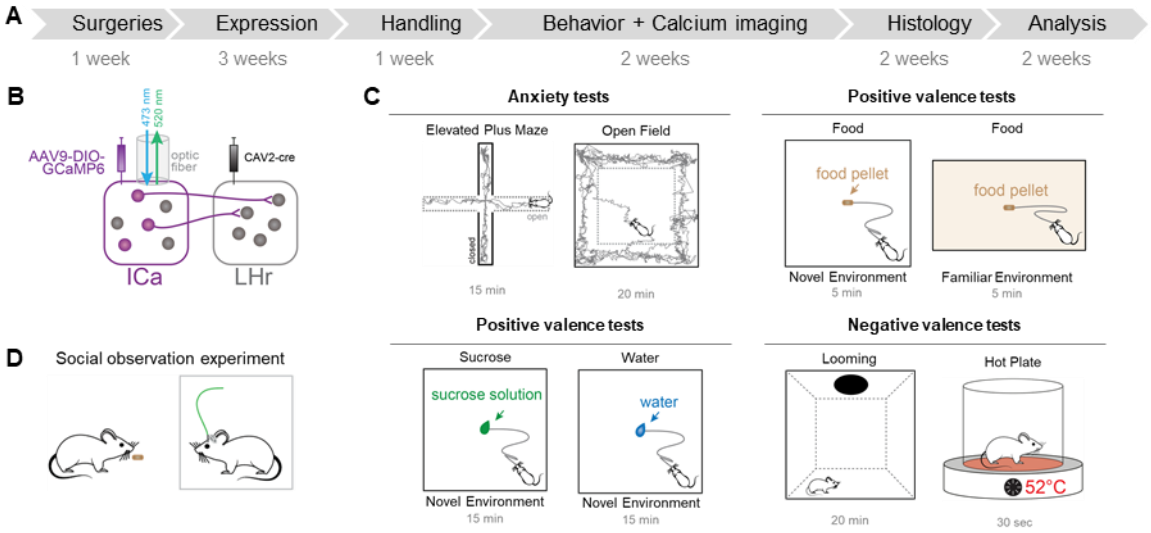


Figure 14. Fiber photometry recordings of the activity of the anterior IC to LH neurons during behavioral assays. A: Timeline of the experimental project. **B**: Representation of the surgical procedure including virus injections and optic fiber implantation sites. **C, D**: Representations of the behavioral tests performed for the first (**C**) and second (**D**) part of the project.

Signal recordings

The optical fiber implanted in the ICa transmits the light emitted by the LEDs to excite GCaMP6m's chromophore. The 20x objective is connected to a camera that detects the fluorescence level, which is indicative of the GCaMP6m activity *in vivo* (Figure 15).

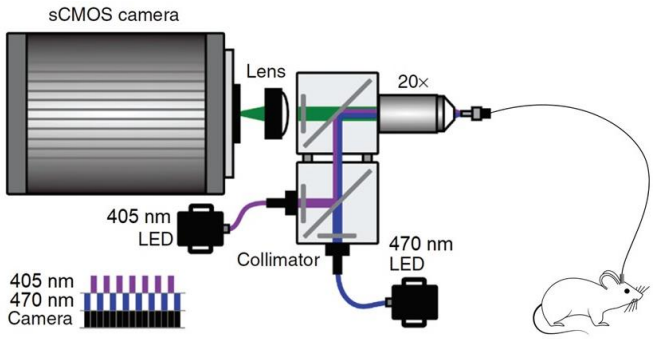


Figure 15. Fiber photometry system. Representation of the microscope used for Ca^{2+} recordings. The diagram at the lower left shows the time-division multiplexing scheme for simultaneous imaging of GCaMP6m at 470 nm and 405 nm (Adapted from Kim et al, 2016).

The optimal wavelength for GCaMP6m excitation is 470 nm, while the isobestic wavelength is 405 nm. This wavelength functions as a negative control, since it is not dependent on Ca^{2+}concentration, to remove motion-related artifacts and signal unrelated to neuronal activity. So, the signals obtained at 470 nm shall be normalized to the 405 nm signals[160]. A Matlab script was used to synchronize each image recording made by the camera, and the GCaMP6m light excitation made by the LEDs (470 nm and 405 nm).

Behavioral assays

The general goal of this project is to understand if, and how, the ICa-LH neurons are involved in anxiety-related behaviors, in valence, and in social observation. To do so, we performed a series of behavioral tests. One week before the experiments, the animals were handled for 30 minutes a day, twice a day, and habituated to being connected to the optic fiber. The tests were performed during the dark phase of the reversed light/dark cycle, using red light to observe the behavior (15 ± 3 lux near the behavioral setups). Humidity and temperature of the experiment room were controlled at 20 to 24 Celsius degrees and 45% to 65%, respectively.

The behavioral maze was always cleaned with acetic acid 2%, and then distilled water, before each animal session. The animals' weight was measured every day, before the test. For the tests that required it, food or water deprivation was done 20 hours before the end of the experiment, and the litter was always changed to removed food scrubs. In those tests, the animals' weight was measured after 2 hours of free access to food, or water.

One camera on top of the experimental maze was used for all the tests, to track the animal's position. Its video recordings were synchronized with the photometry signal recordings. An additional camera was put on the side of the apparatus, in the tests that involved eating, drinking, or pain response.

Anxiety tests

We used two classical anxiety tests: the elevated plus maze and the open field test. In these tests, the apparatus is divided into an anxiogenic and a not anxiogenic area. The goal is to compare the neuronal activity of the neurons between the different zones. Our initial hypothesis is that the ICa-LH neurons are hyper-activated in anxiogenic spaces.

Elevated plus maze (EPM)

The arena has a plus shape (75 x 75 cm), consisting of one open arm and one closed arm. Each animal was placed at the center of the maze and it was left to explore it for 15 minutes. The ICa-LH neuronal activity was recorded in each arm, to analyze if the neurons are differently active in anxiogenic vs not anxiogenic spaces.

Open field test (OFT)

In this test, the arena is squared (60 x 60 cm), and the anxiogenic space is the center, while the not anxiogenic space are the borders the field. The center is defined as 40% of the totality of the square. Each animal was put initially on the center of the open field, and left to explore it for 20 minutes.

Open field test after food deprivation (OFT-FD)

The OFT was also done after food-depriving the animals, in order to control for interactions of the food deprivation with the behavior of the animals. Specifically, this test was used as a control for the positive valence tests, since it is performed in similar conditions, but without the positive valence stimulus component. Similarly to the OFT, the animals were put in the center of a squared arena (60 x 60 cm) and the neuronal activity was recorded for 20 minutes.

Positive valence tests

All the positive valence tests consist of a 60 x 60 cm squared arena with transparent walls, and a positive valence stimulus (reward) at the center: food, sucrose, or water.

Food in new environment test, after food deprivation

The neuronal activity was recorded during 5 minutes, while the animals explored the apparatus. The food-biting moments were automatically detected via the sound.

Food in familiar environment test, after food deprivation

This test is a control to the food in new environment test. The animals were put on a new cage, with bedding and litter from the home cage, to mimic the familiar environment, and a food pellet was inserted in the center. Similarly to the food test, it took 5 minutes, the animals were food-deprived before the test, and the biting was automatically detected.

Sucrose in new environment test, after food deprivation

The sucrose test is similar to the food test, but using a sucrose solution (15% in tap water) instead of a food pellet in the center of the arena. The sucrose consumption was measured using an LED that blinked each time the animal licked from the port. The test took 15 minutes and the mice were food-deprived before.

Water in new environment test, after water deprivation

This test is in all similar to the sucrose test, except that we use water as the reward, and the animals were water deprived prior to the test. The water licking was equally detected, and the test lasted for 15 minutes.

Negative valence tests

In the negative valence tests, the behavior apparatus was not divided into anxiogenic and not anxiogenic spaces, and different type of stimulus were used.

Looming test

The looming test aims to simulate a flying predator by using a screen (15 inch diagonal) above the arena (43 cm wide, 32 cm deep, 20 cm high) where the animal is put. On the screen, a round dark shape appeared in a white background, increasing in size in a few milliseconds. The traditional response of the mice was to freeze or to escape to a protected place: the corner of the arena.

We used the Python package PsychoPy3[161] to trigger the loom stimulus according to Yilmaz and Meister[162]: 15 cycles, in which the black disk started with a size of 2 degrees of visual angle, increasing to 20 degrees for 250 ms; stayed steady at 20 degrees for 250 ms; 500 ms of pause. This made 1 second of looming. These cycles appeared intercalated with a baseline period of 5 minutes for 4 times. So, the total time of the looming test was 20 minutes.

Hot plate test

In the hot plate test, we measure the latency of the animals to lick their paw, after we put them on top of a heating platform, at 52°C. The animals were removed as soon as they showed a pain response (jumping), or after 30 seconds[163]. This is a relevant test because the insula is known to be involved in pain processing and sensitivity[142].

Social observation experiment

In the second part of this study, we wanted to assess the potential mirror-like properties in the ICa-LH neurons, in a social observation context. To do so, we measured the neuronal activity of this population when an animal sees a conspecific performing an action or experiencing a situation, and when the same animal performs the same action, or experiences the same situation.

For each session, there were two animals: an observer and a demonstrator, and the fiber photometry recordings were made from the observer animal's brain. We did the protocol in three independent contexts: eating, drinking, and getting a footshock. The experiments were made during the active part of the cycle[164] and the observer / demonstrator pairs were always from the same sex and cagemates, since it was suggested that familiarity increases the empathic behavior of mice[35,37,165]. Each session was made in only one row, without disconnecting the animal, and it took 26 minutes (Figure 16).

To be sure that the observer animal was watching the demonstrator, we built a head-fixation setup and used it during the baseline and observation period of each session. The animals were habituated to it two days prior to the experiment, for 10 minutes a day, twice a day[166,34].

The setup was built from the ground up, using pieces from ThorLabs, and parts that were designed in FreeCAD and 3D-printed. We also built a 4-walls protection, using plexiglass, to avoid the demonstrator animal from interacting with the observer.

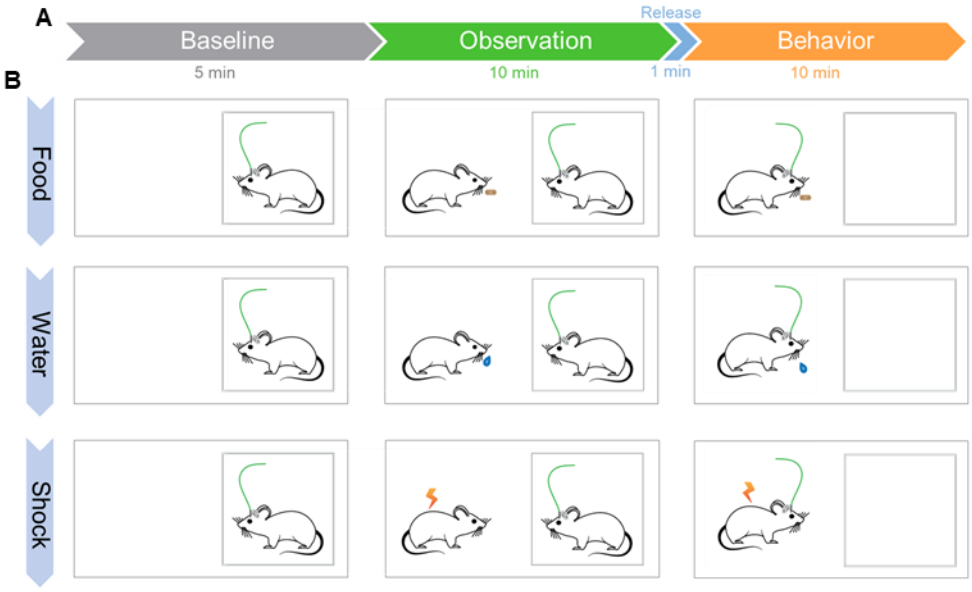


Figure 16: Fiber photometry recording of the activity of anterior IC to LH neurons during a social observation paradigm. A: General timeline of the experiment. **B**: Experimental setup for each condition (food, water, footshock), according to the timeline.

For eating and drinking sessions, the animals were previously food-deprived or water-deprived, respectively. The session started with head-fixing the observer animal, on the arena, connecting the optic fiber, and recording the baseline activity for 5 minutes. Then, we put the demonstrator animal on the arena and let it eat or drink for 10 minutes, while neuronal activity of the observer was being recorded – observation period. After that, we put the demonstrator animal back on the home cage, took the observer out of the head fixation system, and put it on the arena, so that it could eat or drink, for 10 minutes, while neuronal activity was being recorded – behavior period. In the end, the recording was stopped and the observer animal was put back in its home cage.

For the footshock condition, the experimental flow was similar. Instead of using the open arena, we used the footshock box, and instead of letting the animals eat or drink, we delivered a footshock of 0.3 mA for 1 second, every minute, during 10 minutes.

Brain extraction, histology and imaging

After the experiments, the animals were euthanized with pentobarbital (300 mg/kg) and perfused with ringer's solution followed by 4% PFA (antigenfix, F/P0014, MM France) at 4°C in PBS 1x. The brains were extracted and left in PFA overnight at 4°C, for post-fixation. Then, they were transferred to a 30% sucrose solution, in PBS 1x at 4°C, for cryo-protection. After sinking in the sucrose solution, the brains were embedded in optimum cutting temperature compound (OCT) and sliced into 100 µm coronal slices, in a freezing sliding microtome (12062999, Thermo Scientific), and stored at 4°C in PBS 1x, until processing for histology.

Hoechst 33342 staining (1 µg/mL) was performed to stain nucleus for all the slices and images were acquired at the fluorescence microscope (THUNDER Imager 3D Tissue, Leica), using the de-convolution system. Fiber implantation sites in the ICa and viral injection sites in the ICa and LH were imaged with the 10x objective. Viral expression images (cell bodies in the insula and axonal fibers in the lateral hypothalamus) were taken with the 40x objective. All pictures were overlayed using the coronal Paxinos atlas (4th edition).

Data analysis

After the behavioral tests, the photometry recordings were analyzed using a custom Matlab script. The first step was to normalize the 470 nm and 405 nm signals, by subtracting the mean fluorescence from the fluorescence recorded by the fiber at each time point, and dividing this value by the mean fluorescence $((F-F_{mean})/F_{mean}) = \Delta F/F$. The mean fluorescence was calculated over a 5 minutes sliding time window (and not the whole time of the recording), in order to remove the photo bleaching effect that happens over time.

Then, the Ca^{2+}independent signal (405 nm) was subtracted to the raw GCaMP6m signal (470 nm), to eliminate unspecific fluorescence (including potential movements artifact). The result was the global signal ($\Delta F/F = \Delta F/F_{Ca2+} - \Delta F/F_{isosbestic}$), that was used as an estimate of tonic activity of the neurons (Figure 17A). Finally, the number of Ca^{2+} transients was detected using a threshold to identify them (GCaMP6m peaks > 4% $\Delta F/F$), and interpreted as phasic firing of neurons (Figure 17B). In the end, we had three variables as results: global signal, the transients' frequency, and the transients' amplitude. For some tests we also did an event-based analysis (per-stimulus average), where the signal was averaged and compared between defined periods: before and after event onset. The events could be biting, licking, footshock, or loom onset, depending on the test.

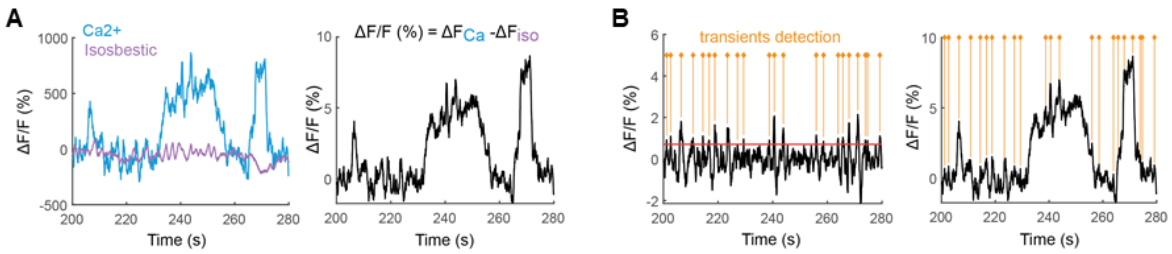


Figure 17. Fiber photometry fluorescence signal processing illustration. A: Example of the global Ca^{2+} signal recording and its correction subtracting the isobestic signal. **B**: Example of the Ca^{2+} transients' detection in the corrected signal trace.

The software Bonsai was used to track the mouse location, which was synchronized with the signal acquisition, allowing us to know the neural activity at any position. Different zones or different time windows were defined for each test. Each parameter (global signal, transients' amplitude and transients' frequency) was averaged, and statistically compared using student paired t-tests. The mean ± SEM was plotted using Matlab or Prism8. Statistical significance was defined at a p-value lower than 0.05. *p < 0.05, **p < 0.01, ***p < 0.001.

When correlation analyses were performed (Figure 20), we calculated the Pearson's correlation coefficient (r^2), that quantifies the magnitude of the correlation between both variables[167]. The closer it is to 1, the stronger the correlation.

RESULTS

We used male and female animals for all experiments. In all cases, when analyzed separately, the results were the same. That is, when there were differences in the neuronal activity recorded between different conditions, those changes were also noticed for each sex. Similarly, when there were no differences in the parameters that were compared for both sexes together, there were also no differences for male and female independently. With this, we can suggest that the ICa-LH neurons are not relevant for the sex differences that have been reported in anxiety[168].

Histology

After the fiber photometry recordings, the animals were perfused and their brains were sliced and imaged under a fluorescence microscope. We took images of the LH and ICa, in order to verify if the stereotaxic surgeries were performed correctly. During the surgery, we injected a viral vector encoding GCaMP6 in the ICa and a viral vector encoding Cre-recombinase in the LH, and implanted an optic fiber above the ICa.

We overlayed the images taken with the microscope with the corresponding brain atlas figures, and marked fiber implantation sites in the ICa in the center of the highest GCaMP6m expression, and the injection sites in the LH (Figure 18).

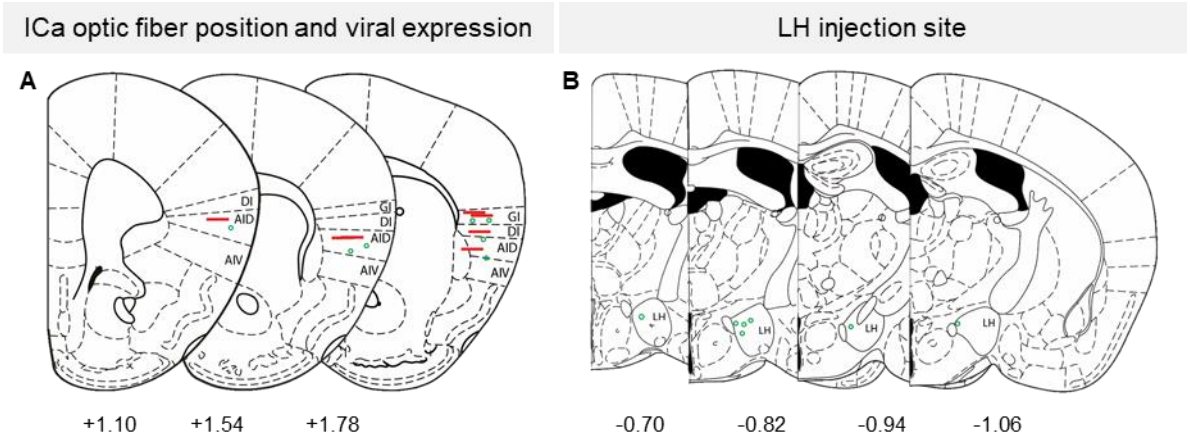


Figure 18: Histological verification of viral injections and optic fiber placement. A: Red bars represent the placing of the optic fiber tip in the ICa. Green circles represent the center of highest GCaMP6m expression. **B**: Green circles represent the injection site in the LH. AID: agranular insula (dorsal) AIV: agranular insula (ventral), DI: dysgranular insula; GI: granular insula. Antero-posterior levels (distance from Bregma, in millimeters) are indicated at the bottom of each brain slice.

It is important to mention that in Figure 18, only the animals where the ICa and the LH were correctly targeted are represented. This corresponds to 7 animals, out of the total of surgeries I performed. In some results, n is higher than 7 because we used data from a previous member of the lab, that performed the same experiments.

Activity of ICa-LH neurons in anxiety and valence tests

To investigate the role of neurons of the anterior insula projecting to the lateral hypothalamus (ICa-LH) in anxiety and valence-related behaviors, we performed fiber photometry recordings in a set of behavioral assays. As explained in the Materials and Methods section, we recorded neuronal activity in classical anxiety tests (open field test and elevated plus maze – Figure 19 and Figure 20), positive valence tests (food, sucrose, and water in new environment – Figure 21 and Figure 22) and negative valence tests (hot plate and looming test – Figure 23).

The activity of ICa-LH neurons is independent of anxiogenic properties of the environment

In the OFT and EPM, which are classical anxiety tests, the animals spent 20 minutes and 15 minutes exploring the two apparatus, and were able to freely cross between their anxiogenic and not anxiogenic spaces. In the OFT, the border is a safe environment while the center is anxiogenic. In the EPM, the closed arm is a safe environment while the open arm is anxiogenic.

The Ca^{2+} signal of each animal was quantified during the whole test time. Individual representative traces (Figure 19A and D) suggest the global signal is not different between anxiogenic and not anxiogenic zones. Additionally, the transient activity (Ca^{2+} signal peaks) appears similar regardless of the zone of the apparatus where the animal is at each moment.

This observation was consistent when we averaged the data across animals (n=9). The heatmaps (Figure 19B and E) visually represent how the Ca^{2+} signal intensity varies in the behavior apparatus, on average, for all animals. They also do not indicate a different activation pattern between the border and center, nor between the closed and open arm.

In fact, there were no statistically significant changes (p-value indicated in the Figures) of the global Ca^{2+} signal when the animals were in the anxiogenic areas (first bar plot of Figure 19C and F) compared to the safe areas. There were also no significant differences in the transients' amplitude or frequency, between the different zones, in either of the tests (second and third bar plot of Figure 19C and F). In summary, ICa-LH neurons are not hyper-activated in anxiogenic spaces of the OFT and the EPM.

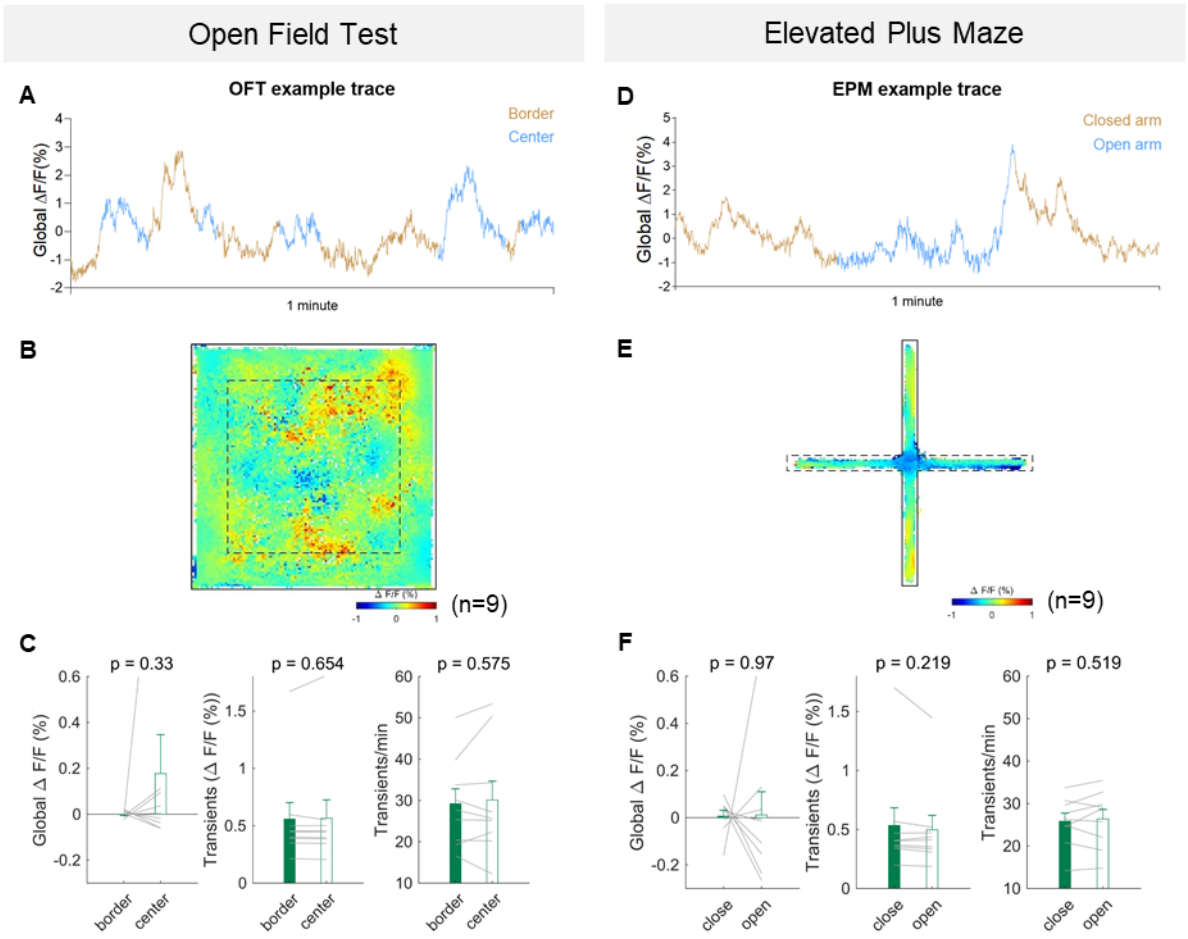


Figure 19: The activity of ICa-LH neurons does not change in anxiogenic spaces. A-C: Open field test (OFT) results (n=9). **A**: Example trace of the Ca^{2+} signal during 1 minute of the test. **B**: Heatmap representing the averaged global Ca^{2+} signal for all mice. **C**: Bar plots representing the global signal intensity, the average transient amplitude, and frequency in the border versus the center of the arena (paired t-tests). **D-F:** Elevated plus maze test (EPM) results (n=9). **D**: Example trace of the Ca^{2+} signal during 1 minute of the test. **E**: Heatmap representing the averaged global Ca^{2+} signal for all mice. **F**: Bar plots representing the global signal intensity, the average transient amplitude, and frequency in the closed arm versus the open arm of the maze (paired t-tests).

The activity of ICa-LH neurons does not correlate with individuals' anxiety levels (trait anxiety)

In the previous section, we found that there is no correlation between the activity of the ICa-LH neurons of each individual animal ('state anxiety'), and its location in the mazes (EPM and OFT). However, as humans, mice show inter-individual variability in their basal anxiety level, which is named 'trait anxiety'. Trait anxiety is independent of where the animal is located in the behavior apparatus, but will define how much time the animals will spend in the anxiogenic versus safe spaces. The 'trait anxiety' level of the individuals could affect the contribution of a neural population to 'state anxiety'. Indeed, the ICa-LH neurons involvement in 'state anxiety' could be dependent on the individual levels of 'trait anxiety'.

For example, ICa-LH activity could be independent of 'state anxiety' in animals with low 'trait anxiety' but could participate in 'state anxiety' in animals with high 'trait anxiety'.

To assess this question, we calculated the 'state anxiety' activity of ICa-LH neurons as the difference of global Ca^{2+} signal between the anxiogenic area and the not anxiogenic area. We chose this parameter because it represents a relative signal, which makes it suitable for comparison between animals. The 'trait anxiety' was defined as the relative time spent in the anxiogenic space (the center of the OFT or the open arm of the EPM).

'State anxiety' neural activity and 'trait anxiety' were correlated using the Pearson's correlation coefficient (r^2). Its values indicate that there is no correlation between these parameters (Figure 20). So, the individual anxiety level ('trait anxiety') is not modulated by the 'state anxiety' ICa-LH activity.

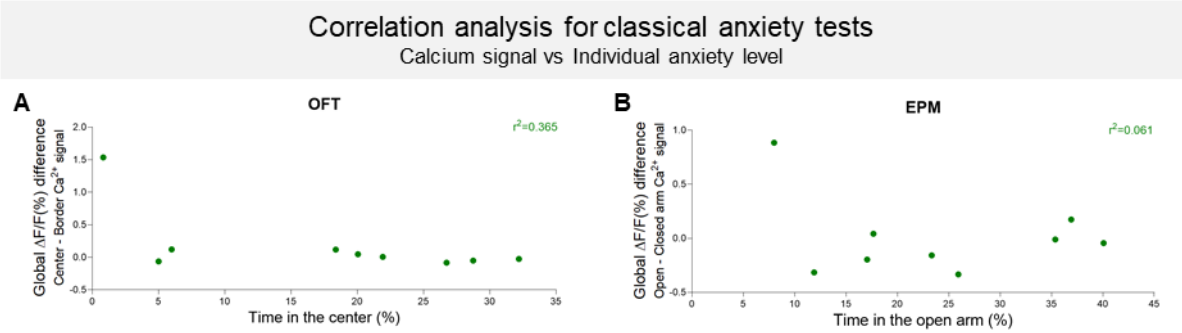


Figure 20: The activity of ICa-LH neurons does not correlate with mice trait anxiety. A: Correlation analysis between the fraction of time spent in the center of the OFT and the global Ca^{2+} signal difference between center and border. **B**: Correlation analysis between the fraction of time spent in the open arm of the EPM and the global Ca^{2+} signal difference between open arm and closed arm. r^2 represents the Pearson's correlation coefficient.

The activity of ICa-LH neurons is lower in anxiogenic spaces in presence of a reward

In the positive valence tests, we used an open arena, similar to the OFT, and put a reward in the center: food, sucrose, or water. This way, we induced an internal conflict in the animals, between avoiding an anxiogenic space, and seeking a reward.

Activity in the reward environment

As it was done for the OFT and EPM, we quantified the global Ca^{2+} signal of each animal during the reward consumption tests, after 20 hours of deprivation. In the food in new environment test, visual analysis of the example Ca^{2+} signal traces suggested a decrease of activity when the mice were close to the food reward (Figure 21A). This tendency is also visible in the average heatmap of the global Ca^{2+} signal (Figure 21B). Indeed, in 6 out of 8 animals, the global signal is lower in the vicinity of the reward. However statistical tests did not reveal a significant decrease (first plot of Figure 21C).

Similarly, it is interesting that there is a significant decrease ($p=0.035$) in the transients' frequency when the animal is near the food pellet, at the center of the arena (third plot of Figure 21C), following the tendency observed for the global signal. However, the

transients' amplitude does not vary between the center and border (second plot of Figure 21C).

Besides food, we used water and sucrose as positive valence stimuli, and performed new behavioral tests. In the sucrose test, the example Ca^{2+} signal trace already suggests a decrease of activity when the mice were close to the reward (Figure 22A). In this test, we observed a significant decrease in the global Ca^{2+} signal ($p=0.009$, visually represented in Figure 22B) and in the transients' frequency ($p=0.023$) when the animals were in the center of the arena, around the sucrose (Figure 22C).

In the water test, the number of animals was lower, and we did not observe statistically significant differences. Nevertheless, a decrease in the Ca^{2+} signal near the reward was observed in individual Ca^{2+} signal traces (Figure 22E), and for the whole experimental group in the heatmap (Figure 22F). There is a decrease in the global signal in the center of the field, in all 5 recorded animals (first plot of Figure 22G), and 4 out of 5 of the individuals show a decrease in the transients' frequency in the center (third plot of Figure 22G).

The results from all the positive valence tests are coherent with each other. Although statistical significance is not always observed, there is always a clear decrease in the global Ca^{2+} signal (tonic firing) and in the transients' frequency (phasic firing) when the animals are at the center of the arena, near the positive valence stimulus (food, sucrose, or water). This suggests that the ICa-LH neurons are under-activated in anxiogenic spaces in presence of a reward.

Control for food deprivation and environment novelty

It could be possible that: (1) the ICa-LH neurons are under-activated in presence of a reward, independently of the anxiety-driven nature of the behavioral apparatus, or (2) the ICa-LH neurons are under-activated in anxiogenic spaces after food- or water-deprivation, independently of the presence of the reward. To eliminate these hypotheses, we performed two control tests: (1) to test if the decreased activity is due to the presence of the reward alone, we recorded the ICa-LH activity during food consumption in a familiar environment (home cage), after food deprivation as well (Figure 21E–H), (2) to test if the decreased activity is due to the anxiety factor after food deprivation, we performed again the OFT test, after food depriving the animals (OFT-FD, Figure 21I–K).

In the OFT-FD, there is the anxiogenic area, but not the positive valence stimulus (food), while the food in familiar environment test has the positive valence stimulus, but not the anxiogenic area. In both cases, we saw no changes in the signal at the center versus the borders of the behavior apparatus (Figure 21G and K). This suggests that the decrease of activity seen in the food / water / sucrose in new environment tests is due to the combination between the anxiogenic effect of the center of the arena, and the presence of the reward in it.

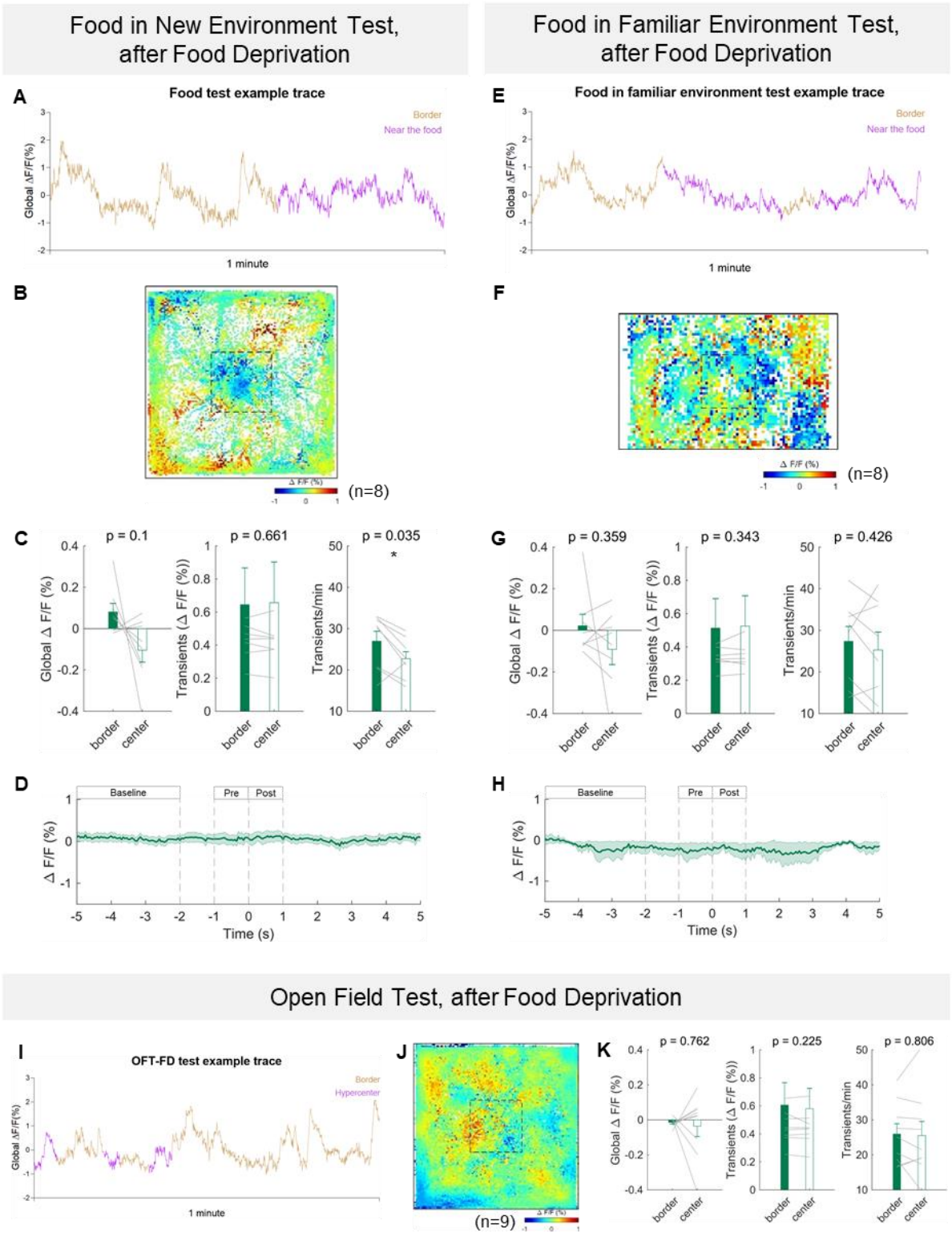


Figure 21: ICa-LH neurons are less activated in anxiogenic spaces, in presence of food. A-D: Food in new environment test results (n=8). **A:** Example trace of the Ca^{2+} signal during 1 minute of the test. **B:** Heatmap representing the averaged global Ca2+ signal for all mice. **C:** Bar plots representing the global signal intensity, the average transient amplitude, and frequency in the border versus the center of the arena (paired t-tests). **D:** Averaged global signal before and after the biting onset. **E-H:** Food in familiar environment test results (n=8).

E: Example trace of the Ca^{2+} signal during 1 minute of the test. **F:** Heatmap representing the averaged global Ca2+ signal for all mice. **G:** Bar plots representing the global signal intensity, the average transient amplitude, and frequency in the border versus the center of the arena (paired t-tests). **H:** Averaged global signal before and after the biting onset. **I-K:** Open field after food deprivation test results (n=9). **I:** Example trace of the Ca^{2+} signal during 1 minute of the test. **J:** Heatmap representing the averaged global Ca2+ signal for all mice. **K:** Bar plots representing the global signal intensity, the average transient amplitude, and frequency in the border versus the center of the arena (paired t-tests).

Control for reward consumption

We also performed an event analysis in all the positive valence tests. The averaged Ca^{2+} signal was plotted before and after the onset of the reward consumption (food biting, water or sucrose licking). This analysis is useful to understand if the ICa-LH neurons are modulated by the specific act of eating or drinking, in each tests' conditions.

There was no change in the averaged global Ca^{2+} signal before versus after the onset of the food biting (Figure 21D), sucrose licking (Figure 22D), nor water licking (Figure 22H), in the new environment tests. This can suggest that the signal decrease we see is a general response to the presence of the stimulus in the new space, and not to the act of eating or drinking itself.

We also did not observe changes in the averaged global Ca^{2+} signal before versus after the onset of the food biting in the familiar environment test (Figure 21H). This indicates that the ICa-LH neurons are really not responsive to the act of eating, and its under-activation is exclusive when the stimulus is in an anxiogenic area.

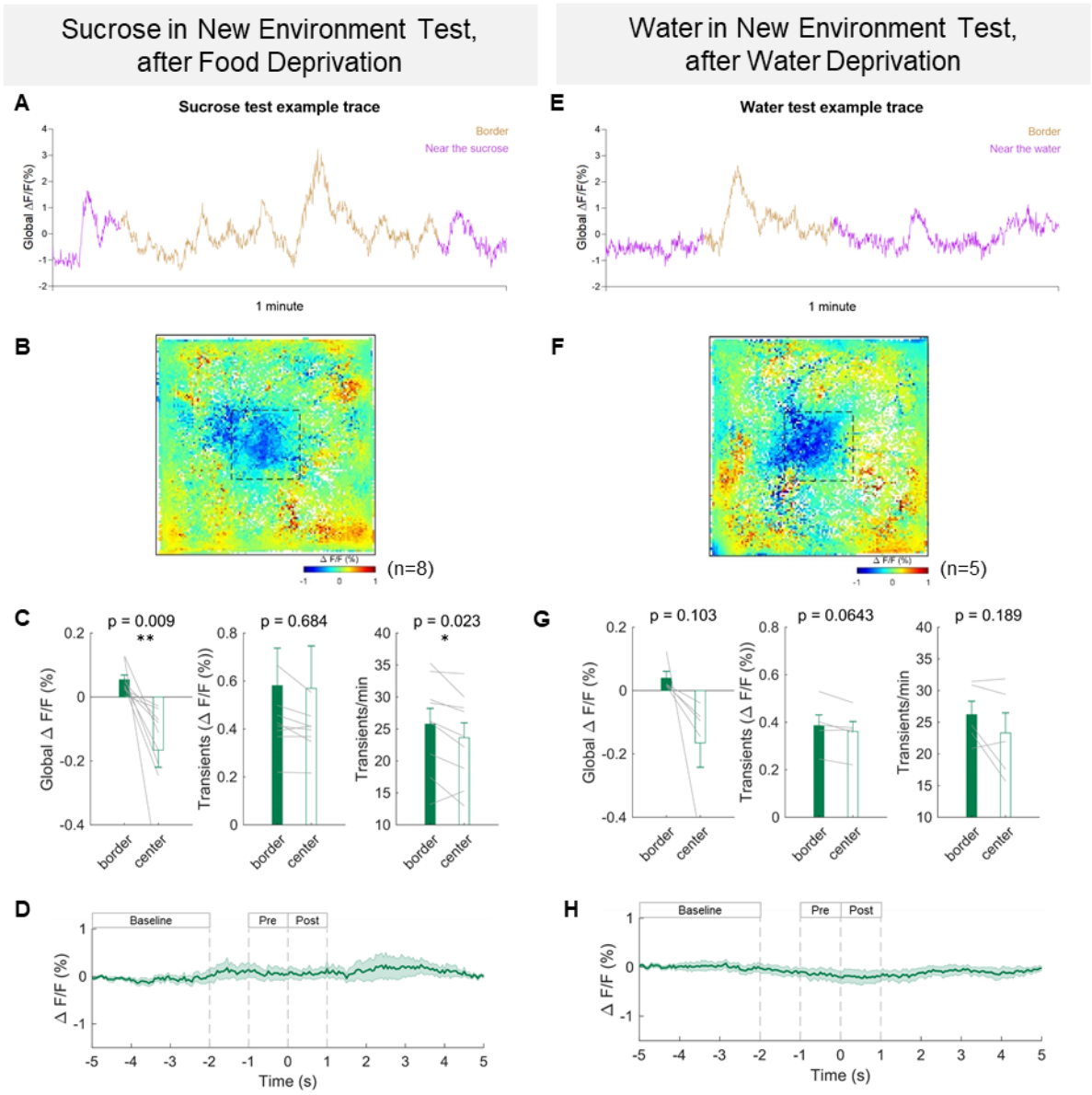


Figure 22: The ICa-LH neurons are lessactive in anxiogenic spaces, in presence of sucrose or water. **A-D**: Sucrose in new environment test results (n=8). **A:** Example trace of the Ca^{2+} signal during 1 minute of the test. **B:** Heatmap representing the averaged global Ca^{2+} signal for all mice. **C:** Bar plots representing the global signal intensity, the average transient amplitude, and frequency in the border vs the center of the arena (paired t-tests). **D:** Averaged global signal before and after the licking onset. **E-H**: Water in new environment test results (n=5). **E:** Example trace of the Ca^{2+} signal during 1 minute of the test. **F:** Heatmap representing the averaged global Ca^{2+} signal for all mice. **G:** Bar plots representing the global signal intensity, the average transient amplitude, and frequency in the border vs the center of the arena (paired t-tests). **H:** Averaged global signal before and after the licking onset.

Activity of Ica-LH neurons does not change with the presence of a negative valence stimulus

The negative valence tests we performed were the hot plate test and the looming test. In these, the apparatus does not have a distinction between anxiogenic and not anxiogenic zones, so we performed only an event-based analysis. However, for the hot plate test, we are not able to show results, because the quality of the signal was not adequate in any of the animals tested.

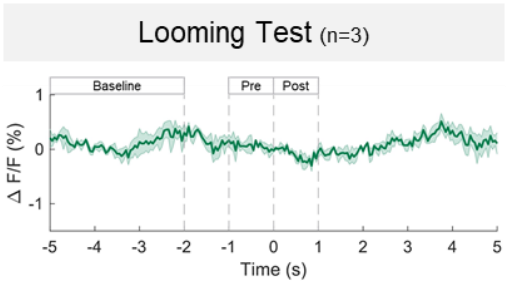


Figure 23. The ICa-LH neurons are not selectively activated during the looming test. Averaged global Ca^{2+} signal 5 seconds before and after the loom onset.

For the looming test, we validated that the loom was well perceived by the animals, by evaluating their behavior before versus after the stimulus appearance. All the animals tested exhibited freezing behavior in response to the looming (data not shown).

We plotted the averaged signal before and after the loom onset, and we did not see changes (Figure 23). The ICa-LH activity is not significantly different immediately before nor after the looming onset.

ICa-LH neurons activity in a social observation paradigm

The second objective of this project was to perform a preliminary study on the activity of the ICa-LH neurons during social observation. We designed a paradigm to study the potential differences or similarities in the neuronal activity when an animal sees a conspecific performing an action or experiencing a situation, and when the same animal performs the same action, or experiences the same situation. If there is similar neuronal activity in observation versus behavior, we can suggest that these neurons have mirror-like properties.

The recordings were made in the ICa-LH neurons, like we did in the first part of the project. The observer animal's ICa-LH neurons were recorded during the session, which was composed of three time epochs: baseline, observation, and behavior. We performed the experiment in three independent contexts: eating, drinking, and pain (receiving a mild footshock, see Methods).

The results were analyzed independently for each session and the parameters analyzed were the global Ca^{2+} signal, transients' amplitude, and transients' frequency - plotted for each time epoch. We also performed an event-based analysis, where the signal was averaged for the 5 seconds before and after the onset of food bites, water licks, or footshocks (for each of the three sessions).

Our working hypothesis was that the ICa-LH neurons have mirror-like activity, which would be characterized by a similar degree of activity between observation and behavior, both different from baseline. Due to the few data collected for this experiment, the results obtained (Figure 24) do not allow us to confirm the hypothesis.

In the food session (Figure 24A and B), the average global signal over the three time epochs (Sup. Figure 2 and Sup. Figure 5) was very low, and stable between baseline, observation and behavior. The transients' amplitude and frequency were also similar between the three time windows (Figure 24A).

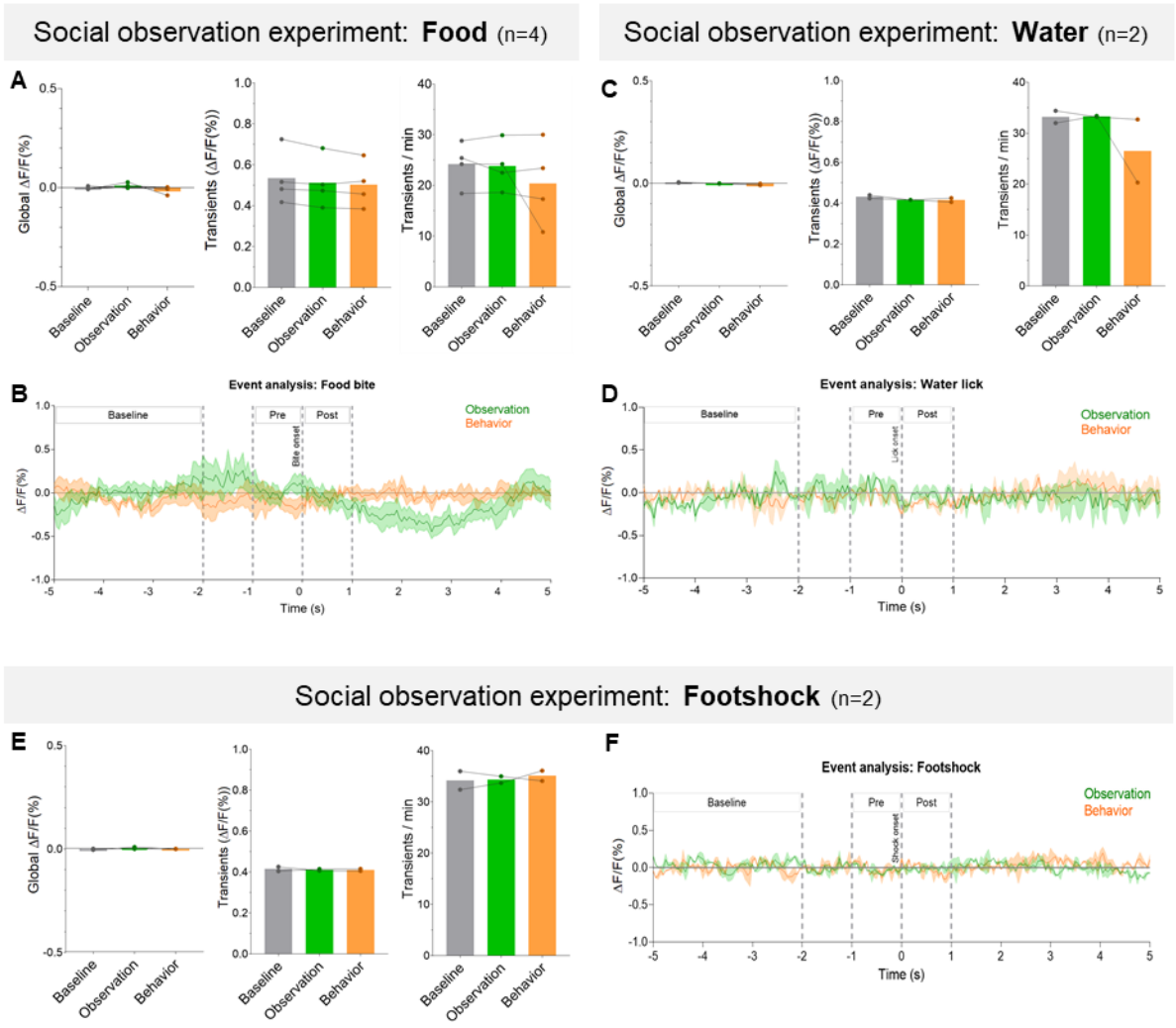


Figure 24: ICa-LH neurons do not show mirror-like properties in a social observation paradigm. **A-B**: Food experiment. **A**: Bar plots representing the global signal, transients' amplitude, and transients' frequency during baseline, observation, and behavior periods. **B**: Averaged global signal before and after the biting onset for observation and behavior period. **C-D**: Water experiment. **C**: Bar plots representing the global signal, transients' amplitude, and transients' frequency during baseline, observation, and behavior periods. **D**: Averaged global signal before and after the licking onset for observation and behavior period. **E-F**: Footshock experiment. **E**: Bar plots representing the global signal, transients' amplitude, and transients' frequency during baseline, observation, and behavior periods. **F**: Averaged global signal before and after the footshock onset for observation and behavior period. Footshock duration: 1 second.

As averaging the entire epoch is not reflecting transient neural responses, we also performed a peri-event analysis (Figure 24B), where we plotted the averaged signal right before and after the food bite onset for the observation period (when the observer watched the demonstrator eating) and for the behavior period (when the observer was eating). The

signal remains stable before and after the biting onset during the behavior period. During the observation period, there is a slight decrease, after the biting onset, but it is not significant.

The results from the water session (Sup. Figure 3 and Sup. Figure 5) were similar as the ones from the food session (Figure 24C and D). The global signal, as well as the transient's amplitude and frequency were not significantly different between behavior or observation, and baseline (Figure 24C). The average of the signal before and after the water licking onset was not different (Figure 24D).

In the same way, in the footshock session (Sup. Figure 4 and Sup. Figure 5), the three signal parameters measured (global, transient amplitude and frequency) were not different between the time epochs (Figure 24E), and the signal did not change around the footshock onset (Figure 24F).

DISCUSSION

The anterior insular cortex is an important structure for the ability to recognize stimuli of positive and negative valence, and its activity is increased in anxiogenic situations. However, our data suggest that the neuronal population projecting to the lateral hypothalamus is not relevant for anxiety-related behaviors.

The ICa-LH in anxiety and valence

In the classic anxiety behavioral tests – EPM and OFT – the Ca^{2+} global signal and Ca^{2+} transients' amplitude and frequency were independent of animals' location in the anxiogenic or not anxiogenic zones. This suggests that the tonic firing and the phasic firing of the ICa-LH neurons are not affected by the anxiety-inducing effects of the OFT or EPM tests. In conclusion our results refute our initial hypothesis, and suggest that ICa-LH neurons are not activated in anxiogenic spaces.

As an additional analysis step, we plotted the averaged Ca^{2+} signal difference between anxiogenic and not anxiogenic space ('state anxiety'), for each animal, as a function of its individual anxiety level ('trait anxiety'). The correlation coefficient values indicated that there is no correlation between the two parameters. Our results therefore suggest that the ICa-LH projection neurons are not involved in the inter-individual variability that exists in anxiety levels.

As mentioned in the Introduction, the initial fiber photometry recordings made in our lab were done in all glutamatergic insular neurons, in the anterior or posterior section. Results show that there is an increased activity in the ICa when the animals were in the anxiogenic spaces: center of the OFT and open arm of the EPM. Since we did not observe any changes for the ICa-LH population, we can conclude these neurons are not responsible for the overall ICa activation in anxiogenic spaces. However, recordings made in the ICa-BLA neurons suggest that this population is involved in this effect.

It is important to highlight that there is no way to directly assess the animals' emotions. For example, one can argue that if the animal is in the open arm at a certain moment, it means it is more anxious, because it is at an unprotected and high space. However, one can also assume that during the same moment, the animal is less anxious, which is why it had the audacity to go to the open arm. For this reason, the best way to quantify the trait anxiety level of each mouse is by calculating the time ratio spent in the anxiogenic space over the whole test time.

Although our results do not suggest a role for the ICa-LH neurons in anxiety-related behaviors, it could still be interesting to analyze the data in a more detailed manner. For example, we could analyze how the signal varies when the animal enters versus when it leaves the open arm of the EPM or the center of the OFT.

Regarding the positive valence tests – food / sucrose / water – we found that the ICa-LH neurons are under-activated in the anxiogenic space where there is the positive valence stimulus. Interestingly, this decrease in activity is not present when the stimulus is in a familiar environment, nor when the stimulus is absent from the anxiogenic space. With this, we propose that the ICa-LH neurons are involved in coding the conflict between the instinct to avoid a new space and the instinct of seeking a reward. The global decrease in the Ca^{2+} signal, however, is not visible when we plotted only its average for 5 seconds before and after the biting or licking onset. Such results indicate that the ICa-LH neurons are responsive to the presence of the positive valence stimulus, but not to the act of consuming the reward (eating or drinking in our experiments).

This can be integrated with previous data collected in our lab (unpublished). Fiber photometry recordings of all glutamatergic neurons of the anterior and posterior insula revealed that only in the posterior insula there is a decrease in the signal around one second before the animal bites the food, and an increase right after biting it. For the anterior insula, however, there was no change in the signal. The lack of variance in the ICa activity can suggest that this sub-region's activity is not modulated by the biting. It can also mean that there are different ICa subpopulations that respond in opposite ways to the biting. For example, one population has an increased activity and the other shows a decreased activity around the biting onset, and they both cancel out each other.

We should also mention that although there are no changes in the event analysis, in the same experiments, there is an increase in the glutamatergic ICa neurons when the animals were near the reward. This indicates that the ICa, as a whole, is hyper-activated in anxiogenic spaces in the presence of positive valence stimuli, but that the ICa-LH projection neurons are not responsible for such effect. Recent experiments in the lab suggest that the ICa-BLA neurons are involved, since they show the same hyper-activation pattern.

From the results obtained in this report, and adopting a circuit point of view, we can suggest that the upstream regions of the ICa-LH population are either inhibiting it more strongly, or exciting it less strongly, in the anxiogenic spaces where the reward is present. As a consequence, the downstream regions of the ICa-LH neurons will receive less neurotransmission. Since the ICa-LH neurons are not widely studied yet, several anatomical approaches could also be used to better understand what are the upstream regions of this

projection, as well as the presence of collaterals. For example, a Cre-dependent rabies virus tracing approach could allow us to elucidate such question.

Interestingly, our results are in line with a recent study on the ICa-LH projection neurons[112], which has shown that this population is activated by aversive stimuli, and causally involved in feeding behavior. Specifically, when the ICa-LH neurons are inhibited, the animals' feeding behavior increases[112]. We suggest that the ICa-LH neurons participate in the capacity to surpass the instinct to avoid a new environment, in order to obtain a reward.

To confirm our results, we could perform other behavioral tests. First we could place the reward (food, sucrose, or water) in the anxiogenic zone of the EPM. Second, we could place the reward in the safe zone of the OFT (border) or of the EPM (closed arms), instead of putting it in the center or open arms, and measure the difference in the Ca^{2+} signal between the different zones, as we did for the other tests that were performed. This would be another negative control, since in this configuration, there is no conflict between the anxiogenic potential of the center / open arm, and the positive valence of the stimulus. Therefore, we would not expect a change in the activity of these neurons.

Regarding the response to negative valence, the negative valence tests performed were the looming and the hot plate. Unfortunately, we were only able to analyze the data from the looming test, since for the hot plate, the light transmission was not well set up and no animal had a good quality Ca^{2+} signal. In these tests, the apparatus is not divided in an anxiogenic and not anxiogenic space, so we can only perform peri-event analysis (paw licking for the hotplate and loom onset for the looming test). In a previous experiment in the lab (unpublished), fiber photometry recordings were performed in all anterior insula and posterior insula glutamatergic neurons. In the hot plate test, the average global signal was plotted five seconds before and after the paw licking onset. Paw licking is a behavior commonly displayed by the animals to relieve the pain from the hot plate. The results showed that only the posterior insula activity increases significantly immediately after the paw licking onset, while the anterior insula activity did not change. This suggested that the ICa-LH projection neurons would also not change activity with this stimulus.

For the looming test, we plotted the averaged global signal before and after the loom onset, and saw no differences. This suggests that the ICa-LH neurons are not involved in the defensive response of the animals, in this particular test. However, since the number of animals is low, and we only have results from one test, we cannot conclude that these neurons are not important for the response to negative valence stimuli in general. The ideal analysis for this test would be to compare the average Ca^{2+} signal with and without the loom, in order to have a more robust conclusion.

The ICa-LH in social observation

The goal of the second part of this project was to understand if the ICa-LH neurons show mirror-like activity in a social observation context. The results obtained do not allow us

to confirm or refute such hypothesis. The ICa-LH activity did not vary between the baseline, observation and behavior period, for any of the sessions (food, water, footshock).

Our data suggests that there is no mirror-like activity in the ICa-LH neurons in the behavior paradigm that was applied. This is a preliminary study, since it was the first time the paradigm was applied in the lab, and a very small group of animals was used. We performed the experiment with five mice, but the number of animals whose results were analyzed was lower, because some had to be eliminated, due to suboptimal light path connections.

It is also important to mention that fiber photometry is not the ideal technique to address this question, as mirror neurons usually consist of a small percentage of the whole population. Thus it is likely that, even if they are present, their activity is not detected when recording the whole population. The ideal approach would be to use a single cell recording method, such as *in vivo* electrophysiology, or single cell calcium imaging, using *miniscopes*. However, fiber photometry could have revealed mirror like activity if the proportion of mirror neurons was important in the population.

In general, one of the main weaknesses of this project is the low number of animals that often decrease our statistical power. This happened because some of the stereotaxic surgeries were not successful. That is, either the ICa or the LH were not well targeted, and the recorded signal was not of good quality.

Moreover, the insula has different topology along the anterior-posterior axis and it may have different functionality along this axis. So, with an increased number of animals, it would be interesting to plot the results as a function of the fiber position in the AP axis. To have more robust results, the experiment should be repeated with a higher number of animals. This would also be highly important to understand the potential existence of sex differences for ICa-LH activity in anxiety and valence.

Perspective: Optogenetics

Fiber photometry experiments provide a correlation between neuronal activity and behavior. From those, we concluded that the ICa-LH projection is under-activated when the animal is near a positive valence stimulus in an anxiogenic area. In order to identify a causal role of a neural population, we need to use optogenetics. With this approach, we can understand if this population is causally involved with the observed behavior, by activating or inactivating it, and analyzing the effects in the behavior.

The planned follow-up experiment would be applying optogenetics in the adequate behavioral tests, depending on the fiber photometry results. Due to the COVID-19 pandemic and subsequent lockdown starting in March, it was not possible to complete that part of the experimental plan. However, we can discuss expected results, based on our fiber photometry recordings.

The fiber photometry experiments show that the ICa-LH neurons are less active in the presence of a reward located in an anxiogenic space. If there is a causal relationship, then, optogenetic inhibition of these neurons in the positive valence tests should increase reward-seeking behavior. This can be measured as the latency to approach the stimulus, which should be lower (***Figure 25*** 25) or as the number of approaches to the stimulus, the time spent eating/drinking, or the quantity of food, water, or sucrose consumed. An optogenetic activation approach could also be possible, in which case we would expect to see a decrease in the reward-seeking behavior.

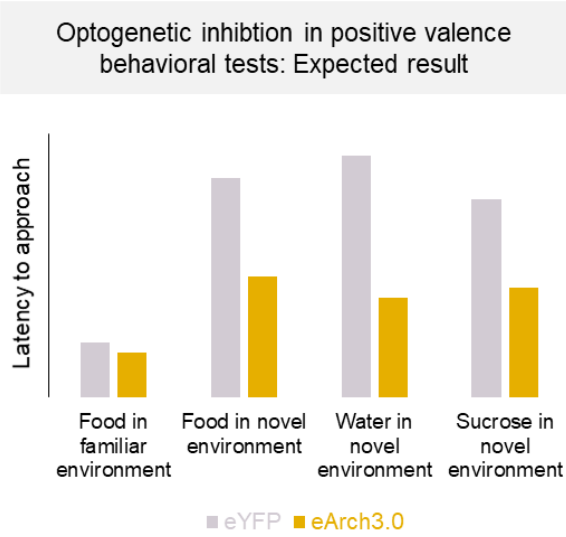


Figure 25: Optogenetic inhibition of the ICa-LH neurons is expected to decrease the reward seeking behavior, when it is in the anxiogenic space. Expected result of an optogenetics inhibition experiment. Latency to approach the reward would be measured in the positive valence tests, between animals injected with a control viral vector (eYFP) and animals injected with an inhibitory opsin (eArch3.0).

As a negative control, to guarantee that the result was a combination of the reward presence with the anxiogenic effect of the center of the arena, we would also perform the experiment during food consumption in familiar environment, like we did with the fiber photometry experiments.

Optogenetic inhibition would be achieved using the proton pump Arch, that leads to the hyperpolarization of the neurons, when excited with 566 nm light[169]. To specifically target the ICa-LH population, we would use a Cre-dependent approach, like we did for the fiber photometry experiments. The vector AAV-EF1a-DIO-eArch3.0-EGFP would be injected in the ICa and CAV2-Cre would be injected in the LH. Half of the animals would be injected in the ICa with the control viral vector encoding only eGFP (AAV-EF1a-DIO-EGFP), and not Arch. For these animals, light stimulation would not induce changes in neuronal activity, which means that any changes seen in behavior were due to the mere presence of the light.

CONCLUSIONS

The principal aim of this study was to understand how the ICa-LH activity is modulated in a series of behavior contexts related with anxiety, valence, and social observation, in healthy animals.

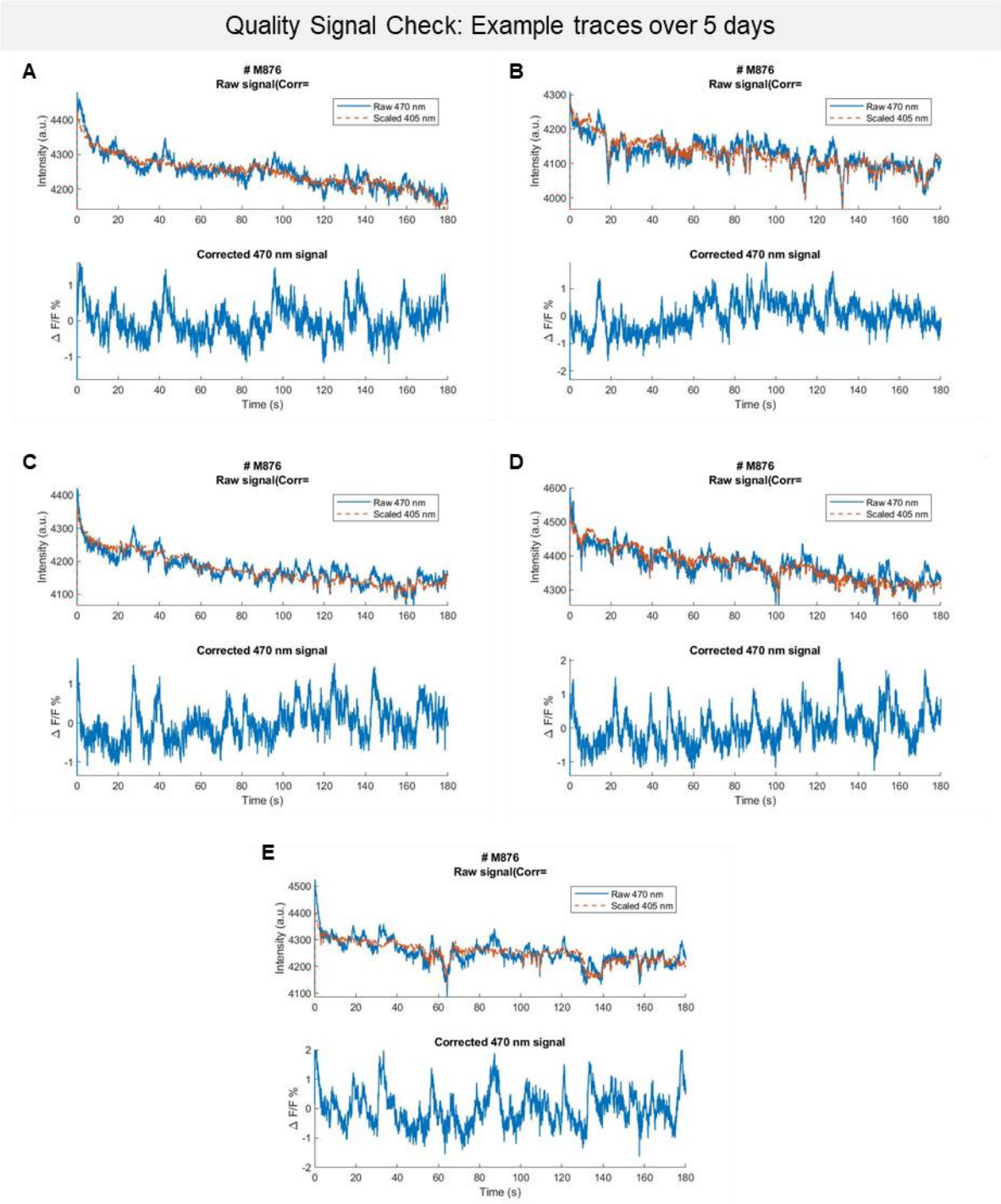
In the first part of this project, we studied how the ICa-LH neuronal activity varies with anxiety-related behaviors, and the presence of positive and or negative valence stimuli, in order to contribute to improve our understanding of the neuronal circuits that are disrupted in anxiety disorders. We found that the ICa-LH neural activity is not modulated during anxiety-related behaviors per se, but is decreased in the presence of positive valence stimuli

in anxiogenic spaces. More experiments would need to be made in order to understand if this projection is causally involved in the recognition of positive valence stimuli in anxiogenic zones.

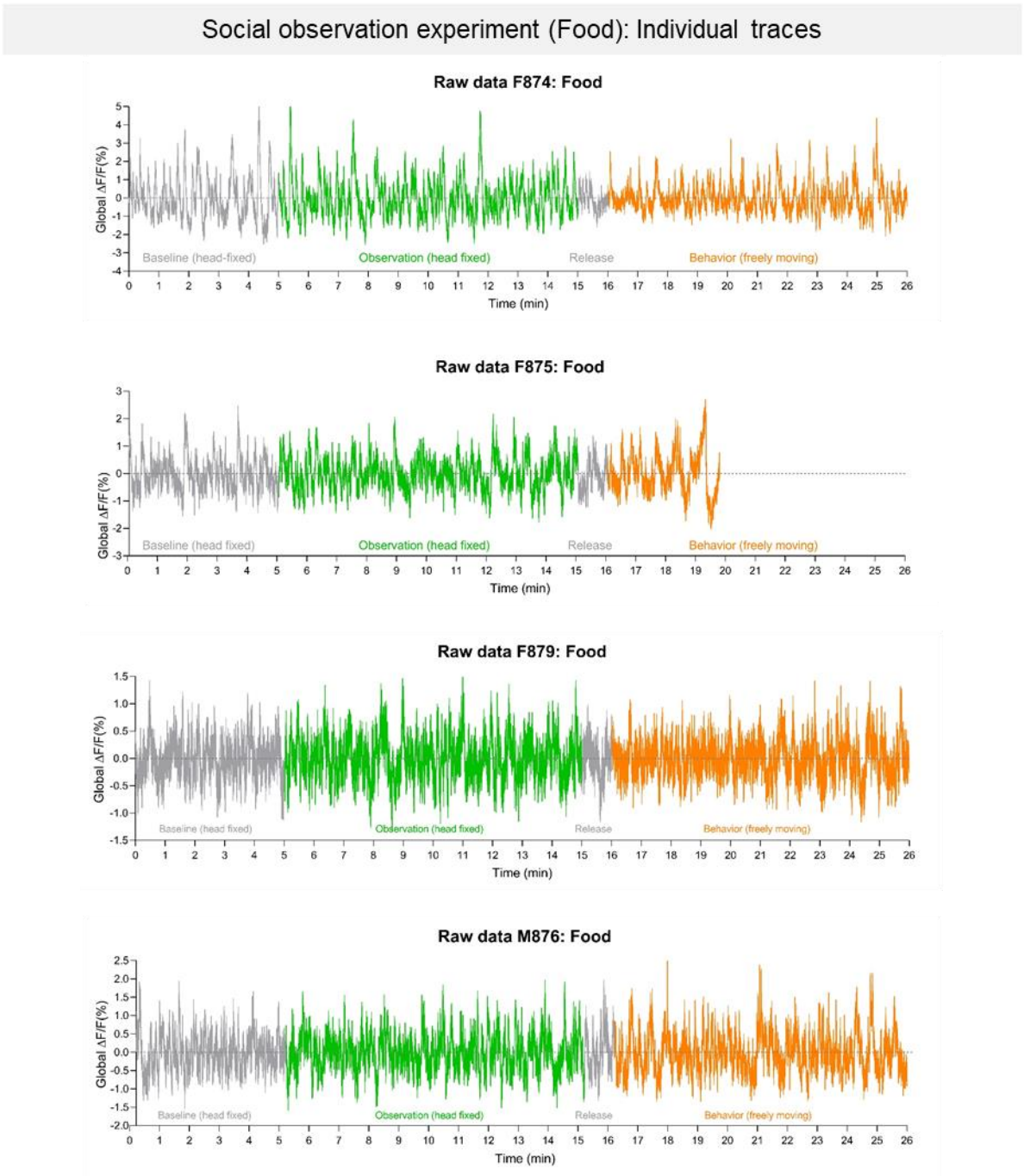
The second goal of this work was to determine if the ICa-LH neurons have mirror activity, in which case we could suggest the existence of mirror neurons, and then use refined single-cell techniques to dissect ICa-LH mirror neurons properties and functions. However, our preliminary results do not allow us to confirm or refute the initial hypothesis.

In summary, this study proposes a role for the ICa-LH neurons in modulating how the positive valence stimuli are perceived in anxiogenic spaces, or how anxiety levels are modulated by the presence of positive valence stimuli. Our data also suggests that this neuronal population is not specifically active in individuals with higher trait anxiety, and it does not have mirror-like properties, in the behavior paradigm we used.

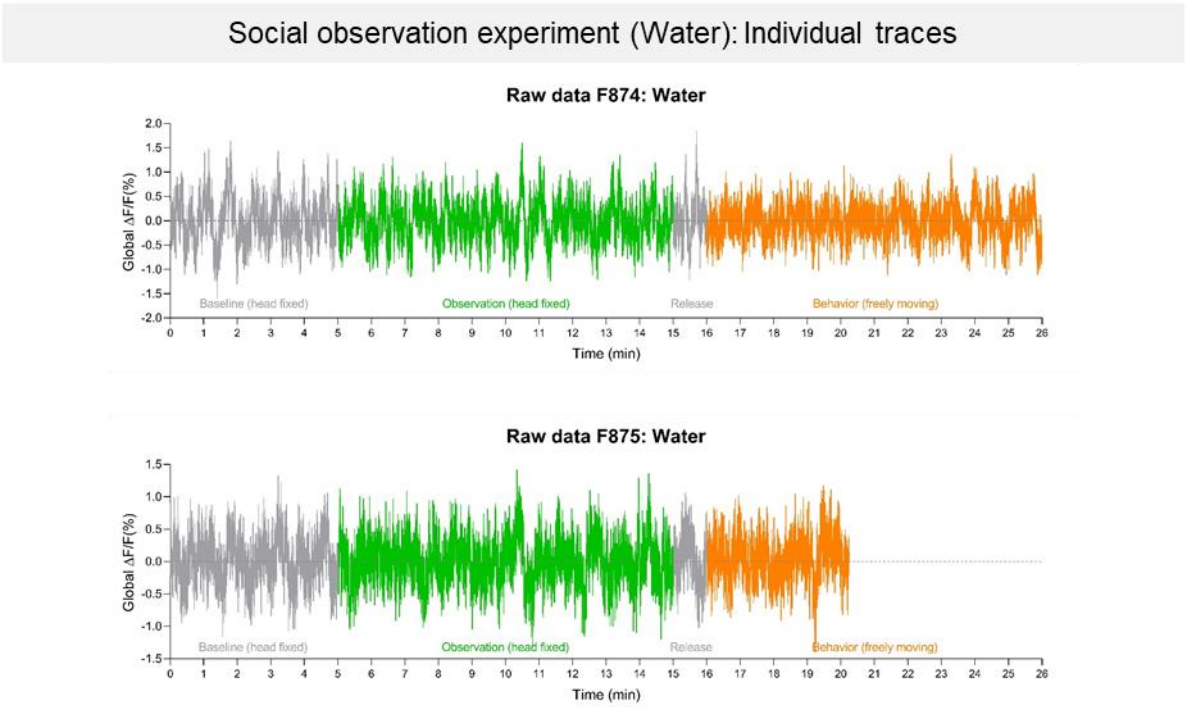
SUPPLEMENTARY FIGURES



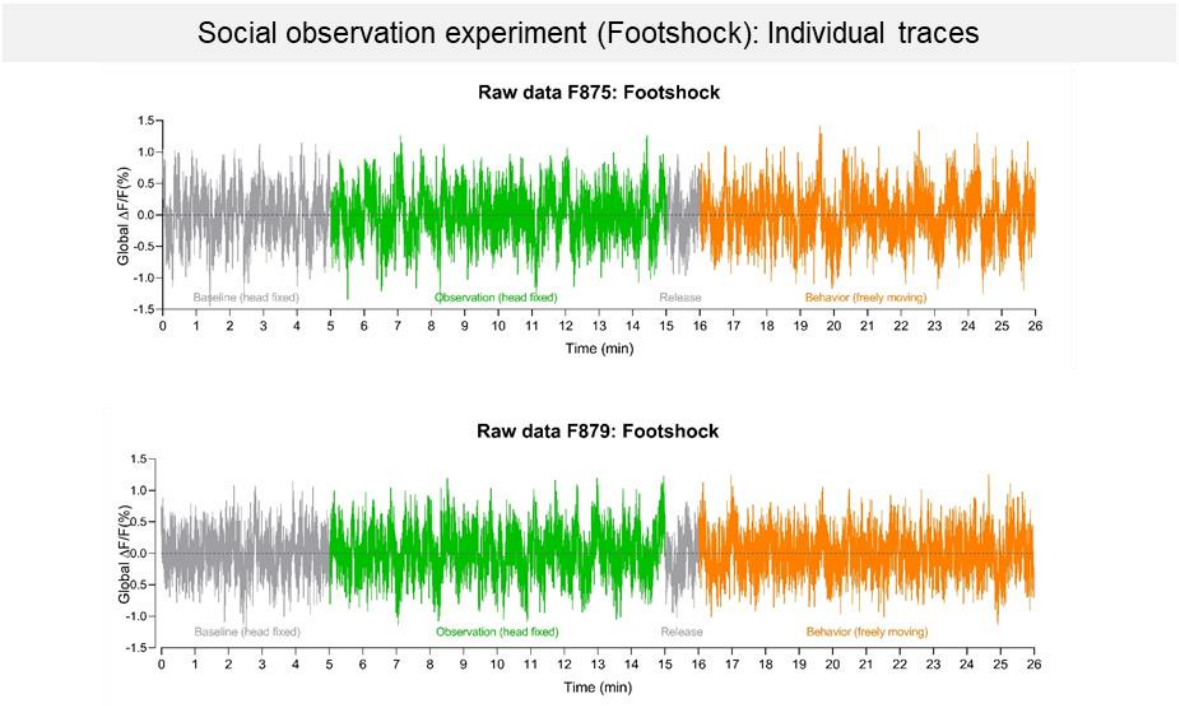
Supplementary Figure 1. GCaMP6m optimal viral expression is 4 weeks after viral delivery. On the third week, after the surgeries for viral delivery, we performed 3 minutes' signal check recordings, to assess which animals were suited for the behavioral experiments. One example is animal M876. From the first day (**A**) of recording to the last (**E**), the signal quality increased (the fluorescence peaks increased from less than 1% to 2%). **A**: First day. **B**: Second day. **C**: Third day. **D**: Fourth day. **E**: Fifth day.



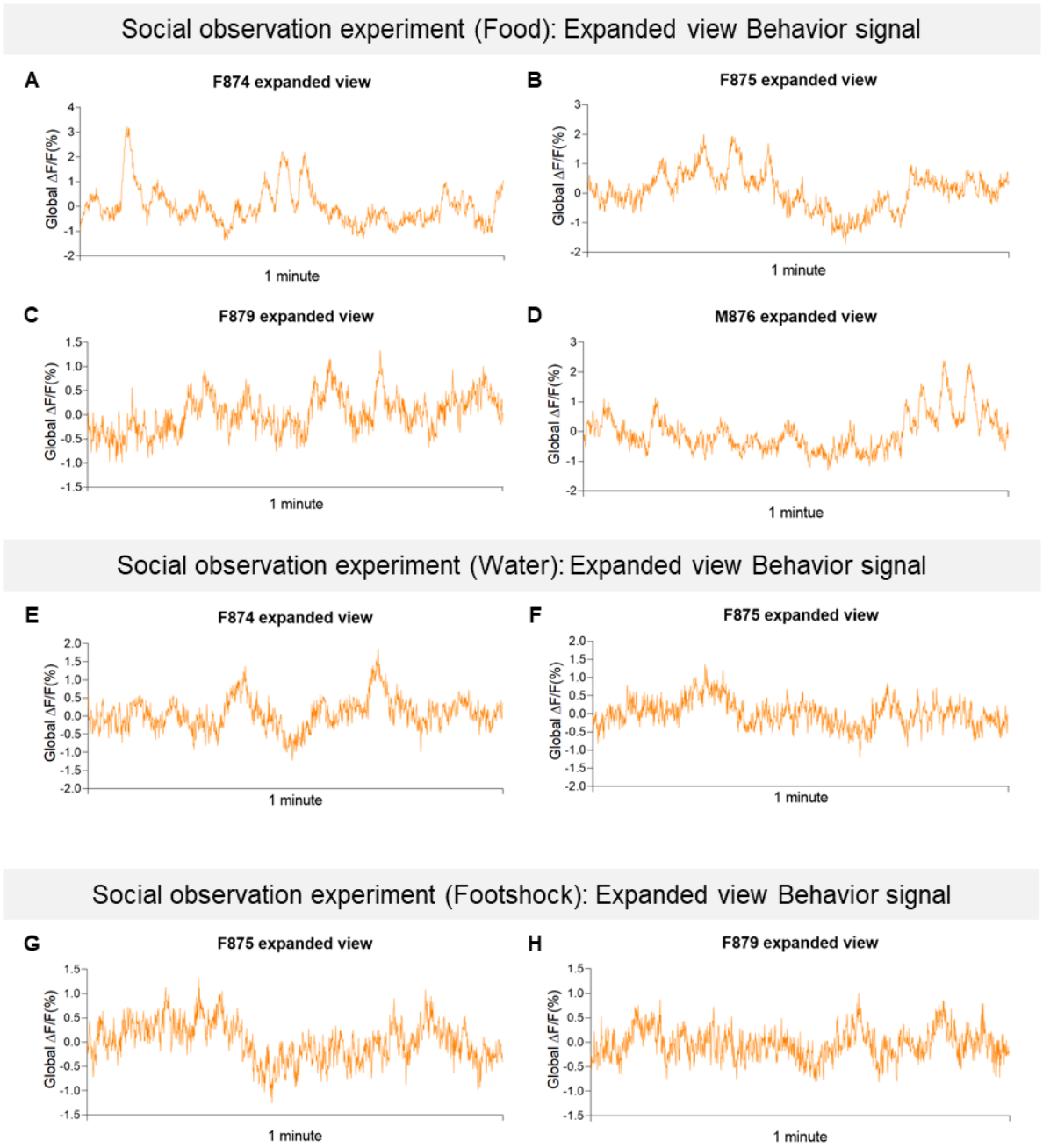
Supplementary Figure 2: Individual traces of the global Ca^{2+} signal for the food session of the social observation experiment. Animals F874, F875, F879, and M876.



Supplementary Figure 3: Individual traces of the global Ca^{2+} signal for the water session of the social observation experiment. Animals F874 and F875.



Supplementary Figure 4: Individual traces of the global Ca^{2+} signal for the footshock session of the social observation experiment. Animals F875 and F879.



Supplementary Figure 5: Expanded view of the individual traces of the global Ca^{2+} signal during random 30 seconds of the behavior period, for all sessions of the experiment. A-D: Food session. **E-F**: Water session. **G-H**: Footshock session.

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