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# DURAÇÃO DA JANELA DE CONSOLIDAÇÃO SISTÊMICA E DINÂMICA DE GENERALIZAÇÃO DE MEMÓRIAS REMOTAS PARA O CÓRTEX INFRALÍMBICO

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# TEMPORAL INVOLVEMENT OF THE INFRALIMBIC CORTEX IN SYSTEMS CONSOLIDATION AND GENERALIZATION DYNAMICS OF A REMOTE AVERSIVE CONTEXTUAL MEMORY

Trabalho de Conclusão de curso apresentado como requisito parcial para obtenção do título de Bacharel em Ciências Biológicas com ênfase em neurobiologia na Universidade Federal do Rio Grande do Sul.

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# OBSERVAÇÃO PRÉVIA

O presente Trabalho de Conclusão de Curso foi escrito em inglês e no formato de artigo para posterior submissão para revisão e publicação na revista Neurobiology of Learning and Memory (NLM). Diversos trabalhos do meu grupo de pesquisa já foram publicados nesta revista (Casagrande, 2018; Crestani, 2018; Popik, 2018; Santana, 2016; Genro, 2012; de Oliveira Alvares, 2008; Oliveira, 2007; Quevedo, 1998; Quillfeldt, 1996, 1997, 1998). A NLM possui fator de impacto 3,01 e é referência em publicações na área de memória e aprendizado.

## ABSTRACT

The transition of short-term memories to long-term memories consists of two serial processes: synaptic consolidation - the process in which memories become more stable and resistant to interference - in the first hours, followed by systems consolidation, that takes place later, in a much larger time scale of weeks. Systems consolidation is the process in which explicit long-term memories become independent of the hippocampus and exclusively dependent on cortical structures for their retrieval. The infralimbic cortex (ILC) participates in remote memory retrieval, although the moment that this structure is effectively recruited for retrieval is not clear. The aim of this work was to characterize the role of the infralimbic cortex (ILC) in the systems consolidation of an aversive contextual memory through the temporary inactivation of this structure with a bilateral intracerebral infusion of $GABA_A$ agonist muscimol, 15 minutes prior to the test. To this end, adult male Wistar rats were trained in the Contextual Fear Conditioning (CFC), a hippocampus-dependent task, and tested in the same or in a different context 2, 14 or 28 days later. We found that the inactivation of ILC 2 and 14 days after training had no effect upon memory retrieval. On the other hand, 28 days after training memory retrieval was impaired, showing the involvement of this structure in remote, but not recent memory retrieval. Besides, animals were tested in a different, yet similar context in order to evaluate memory generalization. This experiment allowed us to find that, 28 days after training, that remote memory was generalized, since the inactivation of ILC led to a decrease of freezing for both contexts.

## 1.1 INTRODUCTION

The retention of information through external experiences constitutes the phenomenon of memory formation (Abel, 1995), which is capable of defining each one's identity. However, the retention of this information does not occur instantaneously. Instead, memory is gradually transformed from a more sensitive state to a more permanent state (Frankland & Bontempi, 2005).

Memories can be stored as short-term memories (minutes to hours) and long-term memories (days, weeks, years). The transition process from short-term memories to long-term memories is named consolidation, where memory turns from a disturbance-sensitive and less stable form to a more stable and resistant to interference form (Abel, 1995). Consolidation occurs at synaptic and systemic levels. Synaptic consolidation consists of creating new connections and reorganizing synapses in circuits. Systems consolidation, on the other hand, is a longer process that involves the gradual reorganization of brain regions that store memory (Frankland & Bontempi, 2005).

This process is constituted by a gradual loss of hippocampal dependence over time, where memories become dependent on cortical structures for retrieval (McClelland, 1995). This occurs by strengthening cortico-cortical connections, making new memories gradually integrated into pre-existing cortical memories. The hippocampus, therefore, temporarily stores new information, while permanent storage depends on the cortical network (Frankland & Bontempi, 2005). Despite the process of loss of hippocampal dependence taking place for different kinds of memory, there are some that do not undergo systems consolidation: thus, declarative and spatial memories, in humans and rats, respectively, seem to permanently dependent upon the hippocampus (Bunsey, 1996).

Memories are never completely accurate and over time contextual details can get lost. Synchronously to the systems consolidation process occurs the generalization process, where memories gradually lose precision and detail. Generalization is an important process in the evolutionary history of animals because it alerts them to potential dangers when they are exposed to situations similar to other previously harmful circumstances (Xu, 2013). In this case, there is a balance between the level of precision and the degree of generalization, which makes the process beneficial to the animal. However, exacerbated generalization is detrimental, and is related to several mental disorders, such as Post Traumatic Stress Disorder (PTSD) and exacerbated anxiety, which can lead to other disorders such as depression (Xu, 2013). PTSD is one of the most common generalization disorders and consists of exacerbated fear of situations that would normally be considered safe as a result of a past traumatic event, leading to hyperarousal and anxiety (Mahan & Ressler, 2012).

Aversive memories generated by trauma are amygdala dependent, which regulates the responses of conditioned fear and associative learning. The amygdala receives projections from the hippocampus and prefrontal cortex (PFC) (Mahan & Ressler, 2012). For a long time it was thought that only the amygdala was the storage place of these aversive memories. However, studies have shown that the prefrontal medial cortex (mPFC) regulates the expression of amygdala-dependent memories, mainly stimulating the extinction process (Vidal-Gonzalez, 2006). The mPFC, nucleus reunens (NR) and hippocampus, in addition to the amygdala, constitute a memory generalization circuit where the NR projects to the hippocampus and back to the mPFC and the hippocampus also to the mPFC, creating a closed-loop with the projection from mPFC to NR (Xu, 2013).

There are three subareas inside the mPFC: the prelimbic cortex (PLC), the infralimbic cortex (ILC) and the anterior cingulate cortex (ACC) (Öngur & Price, 2003). PLC and ILC form a bi-directional mechanism of fear expression modulation, where the first sends excites and the second inhibits amygdala projections (Vidal-Gonzalez, 2006).

Studies using ILC injury have shown that retrieval of aversive memories is impaired when this region is not participating (Quirk et al., 2000; Lebron et al., 2004). In addition, Barrett et al. (2003) showed through metabolic mapping, using a radiolabeled glucose analog, an increased excitability in ILC neurons when animals are retrieving extinction memories. These results suggest that ILC increased activity suppresses fear expression after extinction (Milad et al. 2006; Quirk et al. 2006).

In addition to ILC's role in memory extinction, Torres-García et al. (2017) showed the role of this structure in systems consolidation. In this study, different protocol intensities were used in animal training (1.0 and 3.0 mA) and it was shown that ILC inactivation with tetrodotoxin impaired memory consolidation in animals trained with the moderate protocol (1.0 mA).

In Pavlovian (classical) conditioning a neutral conditioned stimulus (CS) acquires the ability to generate defensive responses when it is paired with an aversive unconditioned stimulus (US) (Wilensky et al., 2000). In the case of contextual fear conditioning (CFC), the aversive unconditioned stimulus is the paw shock and the neutral conditioned stimulus is the context itself.

Fear index of animals is quantified through freezing, which refers to how long the animal remains crouching with no visible body movement, except for breathing (Quillfeldt, 2016). The experiment becomes valid only if the control group learns the task properly, obtaining an increase in the freezing percentage between training and test sessions. This increase indicates that animals learned the task correctly (*ibidem*).

## 2.1 OBJECTIVES

Using CFC, this project aimed to characterize the role of ILC in the dynamics of systems consolidation, seeking to investigate the duration window in which this structure is recruited for retrieval. It was also analyzed its relation with the quality of memory retrieved, allowing to verify the moment between conditioning and test in which generalization occurs.

By applying local pharmacological inhibition through stimulation of GABAergic (inhibitory) interneurons, in distinct intervals between the training and test sessions (2, 14 and 28 days) we intended to understand the ILC and its participation in the dynamics of memory retrieval. Besides, we also intended to investigate the role of ILC in memory generalization, using a 28 days interval between testing and training.

## 3.1 MATERIAL AND METHODS

### 3.1.1 Animals

A total of 74 adult male Wistar rats with 60 days or older (approximately 10 days) were used. The animals weighted 350 to 450 g.These animals were kept in boxes of dimensions 65 x 25 x 15 cm, and on the floor were spread dry and clean wood shavings. Four or five rats per box have water and food available *ad libitum* (the animals fed on Nuvilab pelleted feed), at room temperature between 20 ° C - 22 ° C and under a 12 hour light/dark cycle. The research project was approved by the University's Ethics Committee (Project UFRGS #35.969) and all experiments were performed in accordance with national animal care legislation and guidelines (Brazilian Law 11794/2008). The animals fed on Nuvilab pelleted feed

### 2.1.2 Surgical procedures

The interest structure of this work is the infralimbic cortex, and the coordinates of this structure were adapted from the Paxinos and Watson Atlas (1986), according to the weight range of the animals used. The coordinates

used from Bregma are: AP +0.32 cm, LL ± 0.06 cm and DV -0.40 cm (Santana et al., 2016).

Each animal had its cranial surface exposed through a sagittal incision with No. 20 or 21 scalpel. A bilateral craniotomy was performed using a dental drill at the sites corresponding to the fixed AP and LL measurements. Afterward, two 0.9 cm intracerebral cannulas were positioned, manufactured from a needle with an external diameter of 0.7 mm, caliber 22, and an internal diameter of 0.3 mm. The stereotactic was used for the placement of the cannulas, which were fixed with dental acrylic forming a helmet over the skull bone, closing the bone window produced.

The drugs were infused through a thinner needle (30-gauge “mizzy”), which was introduced into the cannulae. The “mizzy” is 1mm larger than the cannulae because the penetration of the target structure with the thinner needle minimizes local injury.

Animals were anesthetized using a mixture of ketamine (general anesthetic) and xylazine (a sedative /myorelaxant analgesic) administered intraperitoneally (i.p.) at doses of 75mg /kg and 10 mg /kg, respectively. The mixture was administered in a volume of 1 ml /kg.

After surgery animals went through a postoperative period, where they were kept in a heated chamber. For postoperative analgesia, the analgesic and non-steroidal anti-inflammatory drug meloxicam (2mg/kg/24hs) was administered subcutaneously for three consecutive days after surgery, administered in a volume of 1ml / kg. The animals remained in recovery for 5 to 7 days before the beginning of the behavioral tasks.

### 2.1.3 Drugs

Muscimol: it is a psychoactive alkaloid, selective $GABA_A$ receptor agonist with hypnotic and dissociative effects. It binds at the $GABA_A$ binding site and is also a partial $GABA_C$ receptor agonist. When administered locally, it acts by suppressing neuronal activity (Majchrzak & Di, 2000) and is used to temporarily and reversibly inactivate the target structure. The concentration used in this project was 1 µg/µL/side.

Vehicle: DMSO 0,8%.

### 2.1.4 Intracerebral infusions

Muscimol and vehicle (0.8% DMSO) were infused through the mizzy using Hamilton syringes to push 0.5 µL of the drug solution. The needles were inserted into the previously unclogged cannulas and the procedure was repeated on both sides. Infusions were performed 15 minutes before animal testing.

### 2.1.5 Cannulae position verified by histology

Animals that fully recovered from surgery and underwent training, testing and retesting were sacrificed by guillotining and their brains extracted to verify the correct position of the cannulas. 0.5 µL methylene blue was injected into each cannula (same volume used for drug administration).

The brains were dissected and fixed in 4% sucrose paraformaldehyde solution for cryoprotection. Then, they were cut into 50 µm thick coronal sections with a cryostat at - 20 ° C, corresponding to the infralimbic cortex region. The sessions were stained by immersion in hematoxylin-eosin solution and fixed with Canadian Balsam. Finally, they were observed with an optic microscope to determine the position of cannula and "mizzy".

### 2.1.6 Behavioral procedures

Conditioning Session (training): Animals were trained in an automated conditioning box with metal bars through which shock is applied and striped walls. Each animal underwent a habituation of 3 minutes and after that two 0.5 mA shocks were applied, with a 30-second interval between them. Five seconds after the second shock, the animal was collected from the conditioning box and returned to the homecage

Test Session: Animals were tested for five minutes at the context in which they were trained (context A) or in another similar context (context C) to check for generalization. The context C has striped walls, but is circular and has a regular floor.

Retest Session: Animals were retested in the same context where they were tested up to 4 hours after the test to verify their behavior without the drug effect.

### 2.1.7 Experimental design

The effects of muscimol administration on ILC were evaluated 2, 14 and 28 days after training in the same context of conditioning (context A). The effects of muscimol administration on ILC 28 days were also evaluated after training in a similar context (context C). Two groups (muscimol and vehicle) were required for testing at each of the training-test intervals in context A, and for testing in context C. Therefore, there were a total of 8 experimental groups.

A maximum of 11 animals per experimental group was used, for a P = 0.05 and a statistical power of 0.90 (de Oliveira et al., 2008; Genro et al., 2012). In total, 74 animals were used (approximately 9 per experimental group, excluding animals that did not survive surgery).

### 2.1.8 Statistical analysis

After verifying if the data is normally distributed and variances equal, results obtained were expressed as mean ± standard error of the mean (SE). Data were submitted to the two-way analysis of variance (2-way ANOVA), and the effects ordered with the Bonferroni post-hoc test, adopting as a value of statistical significance $P < 0.05$. The data obtained were analyzed and graphs produced using GraphPad Prism ® 7.00 (GraphPad Prism, USA).

## 3.1 RESULTS

**ATENÇÃO**

Os resultados obtidos neste
*Trabalho de Conclusão de Curso*
encontram-se **sob embargo** até serem
aceitos para publicação em periódico
indexado após avaliação pelos pares

## 5.1 CONCLUSION AND PERSPECTIVES

The present work has shown the temporal window of the infralimbic cortex participation in the retrieval of a context-aversive memory. It can be concluded that the ILC role in memory retrieval changes over time, being involved 28 days after the training session, but not at 2 and 14 days (intervals between training and test session). This result is consistent with the hypothesis of systems consolidation and highlights the recruitment of ILC for the storage of systems-consolidated, remote memories. In addition, the present work also showed the role of ILC in the generalization of remote memories, which is consistent with the resulting corticalization of the memory trace after systems consolidation. Thus by inhibiting this cortical area, it was possible to observe a decrease in time of the generalization to the context. In the future, it would be interesting to analyze the dynamics of ILC generalization at intervals of 2 and 14 days after training, in order to investigate whether ILC has a role in the generalization of long-term memories in other training-test intervals.

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