Delving into the claustrum: insights into memory formation, stabilization and updating in mice

Short Title: The claustrum's role in learning and memory

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Abstract

The claustrum is a brain structure that remains shrouded in mystery due to the limited understanding of its cellular structure, neural pathways, functionality and physiological aspects. Significant research has unveiled connections spanning from the claustrum to the entire cortex as well as subcortical areas. This widespread connectivity has led to speculations of its role in integrating information from different brain regions, possibly contributing to processes such as attention, consciousness, learning and memory. Our working hypothesis posits that claustrum neural activity contributes to the formation, stabilization and updating of long-term memories in mice. We found evidence in CF-1 mice of a decline in behavioral performance in an inhibitory avoidance task due to intra-claustral administration of 2% lidocaine immediately after a training session or memory recall. Nevertheless, this does not seem to be the case for the acquisition or retrieval of this type of memory, although its neural activity is significantly increased after training, evaluated through c-Fos expression. Moreover, inhibition of the claustrum's synaptic activity appears to impair stabilization but not the acquisition or retrieval of an unconditioned memory formed in a nose-poke habituation task.

Keywords: mice, lidocaine, claustrum, habituation, memory reconsolidation, memory consolidation, c-Fos

Abbreviations:

CLA: claustrum

GLMM: generalized linear mixed model

HT : nose-poke habituation task

IA: inhibitory avoidance

IHC: immunohistochemistry

IQR: interquartile range

Lido: lidocaine

M1/2: motor cortices

Na: Naïve

PB: phosphate buffer

PBS: phosphate buffered saline

PFA: paraformaldehyde

PV: parvalbumin

RT: room temperature

TR: training

TS: testing

Veh: vehicle

1. Introduction

Memory, a fundamental cognitive process, captivates researchers as they seek to understand its mechanisms underlying formation, consolidation and retrieval (1,2). Within the complex network of the human brain, the claustrum—a nucleus nestled deep within the cerebral cortex—has emerged as a subject of profound interest for neuroscientists studying memory and its neuropharmacology (3,4). This research paper aims to study the participation of the claustrum in memory processes in mice.

The claustrum (CLA), or "hidden place" in Latin, is a thin, irregular sheet of gray matter bilaterally positioned along the insular cortex (5). Structurally, it assumes an elongated shape that spans the rostrocaudal axis of the brain, extending beneath the neocortical mantle and establishing connections with the putamen, globus pallidus and

the extreme capsule (6,7). This positioning emphasizes its potential role as an integrative hub within the brain's vast neuronal network (8).

The CLA's connectivity further highlights its significance, as it possesses extensive reciprocal connections with diverse cortical and subcortical regions. This connectivity allows it to form a network that extends throughout the entire cerebrum (9). Noteworthy, these connections encompass the prefrontal cortex, motor cortex, somatosensory cortex, as well as the visual and auditory association cortices (10–13). Additionally, subcortical connections extend to the basal ganglia (6,7), thalamus (14–16), amygdala (7,17) and hippocampus (18,19), among other regions (20). Such extensive connectivity underscores the CLA's capacity for coordinating information flow across diverse brain regions, suggesting a role in integrating complex sensory, motor and cognitive processes (4,13,21).

Despite decades of investigation, the precise functions of the CLA remain elusive. Its structural characteristics, extensive connections and neurochemical profile position it as a prime candidate for involvement in attention, consciousness, perception and decision-making processes (4,22). Moreover, emerging evidence suggests its contribution to memory formation and retrieval (23–27), thus making it a compelling target for further exploration. The CLA's interactions with memory-related regions, such as the hippocampus and prefrontal cortex, suggest its role in memory consolidation and retrieval (27,28). Additionally, its dynamic connections with the limbic system and its role in mediating attention and sensory integration further support its involvement in memory function (22).

The abundance of functions that the CLA appears to exert is associated with a wide variety of potential dysfunctions. Due to the preponderant role it seems to play in higher cognitive processes, researchers are investigating its involvement in various neurological pathologies, including Wilson's disease (29), epilepsy (30), autism (31), depression, schizophrenia (32,33) and Lewy body dementia (34), as well as neurodegenerative diseases such as Alzheimer’s (35–37) and Parkinson’s (38–40).

Memory consolidation and reconsolidation processes are essential for the stabilization and modification of memories (41–44). Consolidation entails the transformation of newly acquired information into long-term memory through cellular and molecular events such as synaptic plasticity, gene expression and protein synthesis (45). Similarly, memory reconsolidation occurs when a previously consolidated memory is retrieved and subsequently re-stabilized, allowing for potential updates or modifications (46–48). Given the CLA's extensive connections and its involvement in memory-related processes, it emerges as a compelling candidate for influencing both consolidation and reconsolidation phenomena.

In the current investigation, our primary goal is to explore the involvement of the CLA in memory consolidation and reconsolidation processes in mice. To accomplish this, we employed two different behavioral tasks, each presenting diverse motivational and motor challenges. By employing these tasks, we aim to shed light on the CLA's crucial role in learning and memory. This enigmatic brain structure has garnered significant interest and our study seeks to provide valuable insights into its contributions to these fundamental cognitive processes.

2. Results

2.1 The claustrum's role in the acquisition and consolidation of an associative memory

We first studied the claustral neural activity elicited after inhibitory avoidance (IA) conditioning, by assessing through immunohistochemistry (IHC) the immediate early gene c-Fos' expression in this area after the training trial (median step-through latency = 14 sec, IQR = 12.75 - 18.25 sec; (58)). We also included a cohort of behaviorally naïve animals, which served as a control group for the trained set of mice (Figure 1A). To delineate the boundaries of this brain structure, we relied on the concentrated clustering of parvalbumin (PV) labeling, which serves as a defining feature of the CLA (59,60). On top of claustral neural activity, we evaluated c-Fos expression in the CA1 subarea of the

dorsal hippocampus. Numerous studies have suggested that hippocampal subregions are involved in different mechanisms within mnemonic processing (reviewed in (61)), including evidence of impairments observed in contextual memory processing that not only appear to depend specifically on the dorsal hippocampus, but more particularly on the CA1 subregion (62). Furthermore, we investigated neural activity in the motor cortices (M1/2) of the mice, brain regions not previously associated with the processing and encoding of associative memories (Figure 1A and B).

As can be observed in Figures 1B and C, both claustral (CLA, in left panel) and hippocampal (CA1, in central panel) neural activity is significantly increased after the IA training trial ($p < .01$ and .001, respectively, exp. 1 in Suppl. Table 1). On the contrary, the learning experience does not significantly change c-Fos expression in motor cortices (M1/2, right panel in Figures 1B and C; $p > .05$, exp. 1 in Suppl. Table 1).

Figure 1. Neuronal activity induced by IA training. **(A)** We performed IHC on brain sections 90 min after IA training to reveal c-Fos expression in the claustrum (CLA, droplet-like areas in bregma +1 mm slice), CA1 subarea of the dorsal hippocampus (squared areas in bregma -2 mm slice) and motor cortices (M1/2, squared areas in bregma +1 mm slice). We also performed IHC for PV in order to delineate the claustral area in each brain slice. **(B)** Representative CLA, CA1 and M1/2 sections from brain slices of trained (TR) and Naïve mice. The dotted line represents the area evaluated as CLA according to PV IHC. Scale bar represents 50 μm. **(C)** c-Fos density for Naïve and TR animals, evaluated in CLA, CA1 and M1/2 brain slices ($n_{Naïve}$ = 2-13 slices from 4 mice, n_{TR} = 2-14 slices from 4 mice). Small dots represent each brain slice, big dots represent each animal's mean c-Fos density and the group's mean and standard deviation are represented in black lines.

Bearing in mind the significant upregulation of the claustral neural activity due to the training trial of an IA task, we next aimed to study its role in the acquisition of an associative memory resulting from this type of conditioning. We performed bilateral intra-claustral infusion of either a Veh or 2% lidocaine (Lido) solution 1 h prior to the IA training trial, for the purpose of preventing synaptic transmission in this area during learning of this associative memory (Figure 2A). Lidocaine blocks the opening of voltage-gated

sodium channels, leading to a reversible block of action potential propagation (63). As it is shown in Figure 2B, claustral synaptic inhibition before training does not cause a significant effect on the behavioral performance of Lido-administered animals, when compared to control mice ($p > .05$, exp. 2 in Suppl. Table 1).

As previously mentioned, memory consolidation entails the stabilization of newly acquired information into long-term memory (64). To address the role of the CLA in this memory process, we trained two groups of mice and, immediately after training, infused their CLA with either a Veh or Lido solution. After 48 h, their behavioral performance in the task was assessed, as shown in Figure 2C. In contrast to control mice administered with Veh, which presented a higher latency to step-through to the dark compartment, inhibition of claustral synaptic transmission by Lido significantly impaired the performance of mice in this task ($p < .0001$; exp. 3 in Suppl. Table 1).

In order to evaluate whether this impairing effect of Lido on long-term memory was time-specific, we repeated the experiment with another cohort of mice, except for administering either Veh or Lido solutions 3 h after the training trial. As can be observed in Figure 2D, delayed infusions did not affect the mice's latencies to step-through in both experimental groups ($p > .05$, exp. 4 in Suppl. Table 1). These last experiments, depicted in Figures 2C and D, were replicated in female mice, with similar results (Suppl. Figure 1A and B, respectively).

Figure 2. The claustrum in the acquisition and consolidation of an associative memory. **(A)** We bilaterally infused the CLA with either a 2% lidocaine (Lido) or saline solution (Veh) into each hemisphere. Inset: IHC for PV, showing the needle trajectory. Scale bar represents 500 µm. **(B-D, top panel)** We injected mice in the CLA with Veh or Lido solution either **(B)** 1 h prior to the training trial (TR; $n_{Veh} = 8$, $n_{Lido} = 9$), **(C)** immediately after TR ($n_{Veh} = 9$, $n_{Lido} = 8$) or **(D)** 3 h after TR ($n_{Veh} = 8$, $n_{Lido} = 7$). The retention test took place either 2 or 48 h later (TS). **(B-D, bottom panel)** Step-through latencies of animals during TR and TS trials. Median values and IQR are shown in black, individual values in color.

Taking into account the results of this first set of experiments, the inhibition of claustral synaptic transmission impairs the consolidation of an associative memory, while it does not appear to have a significant effect on its acquisition, although its neural activity is significantly increased after training.

2.2 The CLA in the acquisition and consolidation of an habituation memory

Next, we wanted to evaluate whether the CLA is relevant for the acquisition and stabilization of another type of memory, entailing different motivational, sensorial and motor requirements. Therefore, we first trained two groups of mice in a nose-poke habituation task (HT) by exposing them to a hole-board for 5 min, and recorded the cumulative nose pokes throughout time (min 1: $p = .7791$, min 2: $p = .6982$, min 3: $p = .5863$, min 4: $p = .1679$, min 5: $p = .0540$; exp. 5 in Suppl. Table 1; left panel of Figure 3A). Immediately after this training trial, we administered bilateral infusions of either Veh or Lido into the CLA of two groups of mice. As the testing trial progressed, the mice treated with Lido exhibited reduced cumulative nose pokes compared to the control group, indicating impaired habituation to the stimulus (min 1: $p = .3962$, min 2: $p = .3274$, min 3: $p = .0407$, min 4: $p = .0550$, min 5: $p < .0010$; exp. 5 in Suppl. Table 1; right panel of Figure 3A).

Next, we tested whether this effect of intra-claustral administration of Lido in long-term habituation was time-specific. When synaptic transmission in the CLA was impaired 3 h after training, the animals' performance in the task did not present significant differences with control mice ($p > .05$, exp. 6 in Suppl. Table 1; Figure 3B).

As before, we also wanted to study if claustral synaptic activity was necessary for the acquisition of this kind of memory, administering either Lido or Veh 1 h prior to the training trial. This time we extended the trial duration to 15 min, for the purpose of revealing a possible intra-trial habituation of mice to the task. Again, Lido- and Veh-

administered mice did not evidence significant differences in their performance in the task ($p > .05$, exp. 7 in Suppl. Table 1; Figure 3C).

Altogether, this last set of results point to an essential role of claustral synaptic activity in long-term storage of an habituation memory, whereas this area does not seem to be required for the acquisition of this type of memory.

Figure 3. The claustrum in the acquisition and consolidation of an habituation memory. **(A-C, top panel)** We injected mice in the CLA with Veh or Lido solution either **(B)** immediately to the training trial (TR; n_{Veh} = 6, n_{Lido} = 5), **(C)** 3 h after TR (n_{Veh} = 7, n_{Lido} = 11) or **(D)** 1 h prior TR (n_{Veh} = 7, n_{Lido} = 11) of a nose-poke habituation task. When indicated, the retention test took place 24 h later (TS). **(B-D, bottom panel)** Cumulative nose pokes performed by animals through time during TR and TS trials. Big dots represent the group's mean values, small dots stand for individual values and standard errors are shown in black.

2.3 The CLA in the retrieval and labilization/reconsolidation of an associative memory

Next, we set out to address the role of the CLA in memory retrieval. We administered either a Veh or Lido solution 1 h prior to re-exposure to the illuminated platform, for the purpose of preventing synaptic transmission in this area during recall of this associative memory (TS1 in Figure 4A). When tested 24 h later, we observed that claustral synaptic inhibition does not cause a significant effect on the behavioral performance of Lido-administered animals, when compared to control mice ($p > .05$, exp. 8 in Suppl. Table 1; Figure 4A).

Following a reactivation-eliciting reminder, a memory trace can undergo the processes of labilization and reconsolidation, ultimately leading to its re-stabilization with potential updates or modifications (46–48). Therefore, we next sought to study the role of the CLA in the labilization/reconsolidation of an associative memory resulting from conditioning in the IA task. We trained two groups of mice and evaluated their behavioral

performance in the task 48 h later by exposure to the illuminated platform, a reminder that has proven to entail memory reactivation (TS1 in Figure 4B; (57)). Immediately after TS1, we performed bilateral intra-claustral infusion of either a Veh or Lido solution and re-tested their performance 24 h later (TS2). The inhibition of claustral synaptic transmission with Lido significantly hindered the mice's performance in this task, when compared with the control mice given Veh ($p = .0141$, exp. 9 in Suppl. Table 1) and this effect was still present up to 14 d later ($p = .0128$, exp. 9 in Suppl. Table 1; TS3 in Figure 4B). On top of that, this impairment is specific to recall, since Lido-injected mice did not differ in their IA performance to the control animals, when infused 48 h after training and tested 24 h later ($p > .05$, exp. 10 in Suppl. Table 1; Figure 4C).

As previously mentioned, memories are dynamic and subject to influence by various processes like reconsolidation and extinction, which can alter their strength or content (65,66). These processes operate through distinct mechanisms, resulting in different outcomes. Reconsolidation allows for the integration of new information and potential strengthening of the memory, while extinction leads to the formation of a new memory trace with a behavioral response opposite to the original one (67). In this case, the formation of an extinction memory would result in a lower latency to step-through to the dark compartment. Therefore, we aimed to determine whether the diminished behavioral performance observed when synaptic transmission was inhibited after recall (Figure 4B) was either due to an impairment in the reconsolidation of the original memory or a facilitation in the formation of a new extinction memory. Similarly as before, we administered two groups of animals with either Veh or Lido in the CLA immediately after TS1, but evaluated their performance in the task 2 h later, to disclose any possible short-term differences in their behavior. Lido-infused mice did not present significant differences in their step-through latencies compared to control animals, nor did drug-administered mice which did not undergo memory recall ($p > .05$, exp. 11 in Suppl. Table 1; NR-Lido in Figure 4D).

Altogether, the results of this last set of experiments point to a destabilizing effect on memory of claustral inhibition after its retrieval, probably due to an impairment in the reconsolidation process. However, the CLA does not seem to have a crucial role in the retrieval of this associative memory.

Figure 4. The claustrum in the labilization/reconsolidation of an associative memory. **(A-D, top panel)** We injected mice in the CLA with Veh or Lido solution either **(A)** 1 h prior to the first retention test (TS1; n_{Veh} = 10, n_{Lido} = 7), **(B)** immediately after TS1 (n_{Veh} = 13, n_{Lido} = 12), **(C)** 48 h after TS1 (n_{Veh} = 8, n_{Lido} = 6) or **(D)** immediately after TS1 ($n_{TS1\text{-}Veh}$ = 9, $n_{TS1\text{-}Lido}$ = 10) and the third was administered with Lido 48 h after training ($n_{NR\text{-}Lido}$ = 9). When indicated, the second retention test took place either 2 or 24 h later (TS2) and a third test was performed after 14 d (TS3). **(A-D, bottom panel)** Step-through latencies of animals during training, TS1, TS2 and TS3 trials. Median values and IQR are shown in black, individual values in color.

3. Discussion

The claustrum (CLA), a thin, irregular and enigmatic structure located deep within the brain's subcortical regions, has been a subject of growing interest in neuroscience, particularly concerning its potential role in memory processes. In the present study we provide new evidence for a central role of the CLA on memory consolidation and reconsolidation processes using two different tasks on mice.

3.1 Claustral activity and memory consolidation

Neuronal immediate-early gene expression is regulated by synaptic activity and plays an important role in the neuroplastic mechanisms critical to memory consolidation, because it entails the expression of regulatory transcription factors, such as c-Fos. It has been documented that c-Fos expression is triggered in numerous brain regions after either a learning event or memory reactivation (58). Specifically, exposure to a novel environment has been shown to induce c-Fos expression in a significant portion of claustral neurons projecting to the medial entorhinal cortex and certain primary cortices

(68). In our experimental conditions c-Fos expression was determined by inmunohistochemistry 90 min after training on an inhibitory avoidance (IA) task and in naive mice, as a proxy for neural activity. Both the CLA and the CA1 area of the hippocampus, but not the M1/2 cortices, showed an upregulation of its activity, suggesting that the CLA, as it has been proven for CA1 (69), takes part in the information processing related to the learning experience.

Since immunohistochemistry fails to reveal whether the increase in c-Fos expression is a result from learning, memory consolidation or both, we opted for a pharmacological approach. Intracerebral infusion of lidocaine, known for its ability to prevent propagated action potentials through reversible blockade of voltage-gated Na+ channels (70), has been extensively used as an effective method for examining the functional involvement of neural structures in behavior (71–77). We therefore administered lidocaine into the CLA at different time points during two behavioral tasks: IA and nose-poke habituation task (HT). Specifically, lidocaine was administered either 60 min before, immediately after or 180 minutes after both IA and HT training. Performance impairment was observed only when lidocaine was administered immediately after training, as depicted in Figure 2 and 3. It's worth mentioning that the CLA is crossed by fibers originating from the frontal, occipital, and temporal lobes (78). Therefore, one of the potential limitations of inhibiting its neural activity with lidocaine is that it could complicate the interpretation of the results. However, our findings are consistent with those of Terem and colleagues, which suggest that the activity of claustral neurons bearing dopamine receptors is essential for the establishment of cocaine conditioned-place preference and their optogenetic stimulation can induce the development of place preference (26). Moreover, our results align with recent evidence suggesting that CLA stimulation in non-REM sleep enhances the consolidation of a labile associative memory (23).

Post-training administration of drugs influences memory only if given shortly after learning occurs (McGaugh, 1973), and this time-dependent characteristic of pharmacological effects on memory is commonly interpreted as evidence that they impact its storage (McGaugh, 1989). Our results agree with this hypothesis and suggest a critical participation of the CLA on memory storage on both tasks. Additionally, local infusion of lidocaine before training did not impact mice performance when tested shortly afterwards, indicating that its effect may be transient and does not affect attentional or acquisition processes.

Our results reveal a critical role of the CLA in the storage of associative memories in both male and female mice. Given the observed sexual dimorphism in claustral volume and anterior cingulate cortex-projecting CLA cells, as documented in previous studies (79–81), it is imperative to consider sex as a biological variable. As such, we are currently undertaking additional experiments to corroborate our results in female mice. We believe that studying the treatment's response in both sexes leads to more appropriate and balanced application of preclinical studies in translation of results in clinical testing (82).

3.2 The claustrum, memory acquisition and salience processing

When addressing memory processes and their modulation, it is advisable to employ various learning tasks that impose different requirements on the experimental subjects. For this purpose, we utilized IA and HT, where motivational, motor and emotional states differ significantly. Comparable results were obtained in both tasks when the CLA was temporarily inactivated immediately after training, indicating a certain degree of generalization in the types of memories that rely on claustral activity for storage.

One of the main limitations of the IA task is its inability to assess its effects on learning processes, as acquisition occurs in a one-trial step-through situation. Therefore, in our study, the extended 15 minute training in the HT offers a significant advantage

over the IA task, as we can assess intra-trial habituation, providing insight into the rate of acquisition. Under our experimental conditions, lidocaine administered 60 minutes before the HT training trial did not impact the acquisition rate, indicating its inability to impair learning and/or acquisition (Figure 3C). Interestingly, these results align with previous evidence indicating a direct correlation between the neural activity of the CLA and short-term habituation to a non-painful stimulus (83). However, they contrast with findings that indicate CLA neural activity correlated with the acquisition of a delayed eye-blink response (24), as well as with the observations by Terem and collaborators, who noted the selective recruitment of claustral neurons containing dopamine receptors in facilitating the acquisition of cocaine preference (26).

Recent behavioral and physiological findings regarding the CLA suggest that this brain structure plays a vital role in both enhancing resilience to distraction (84) and modulating an organism's vigilance levels in response to varying contextual factors, such as varying degrees of anxiety related to threats (85). Anatomical studies have uncovered robust inputs to the CLA from limbic structures, particularly the basolateral amygdala (84,86). These limbic inputs likely convey "salience signals" to the CLA, which are then transmitted to sensory and motor cortices to coordinate sensory exploration towards relevant stimuli. Consequently, researchers have proposed the CLA as a limbic-sensorimotor interface, facilitating salience-guided orienting during sensory exploration (85). Our results appear to contradict this perspective since no impairment was observed when the CLA was inactivated during training. This suggests an independence of salience processing from claustral neural activity, at least under our experimental conditions.

However, since we did not measure the local concentration of lidocaine in the CLA 60 minutes after its administration, we cannot dismiss the possibility that the inhibition of neural activity at this time point may not be sufficiently intense to affect the aforementioned processes. It has been reported in monkeys that neuronal shutdown

following an injection of lidocaine into the dorsomedial frontal cortex was immediate, occurring within 5 minutes of starting the injection and recovering within 30 minutes (87). However, considering its reported pharmacokinetics and half-life (1.2 ± 0.3 hours after intravenous administration in rats (88)), together with the local administration route, we can infer that lidocaine was likely still present during training. Nevertheless, further studies are required to confirm this assumption.

3.3 The claustrum in memory retrieval, reactivation and restabilization

With the aim of determining the involvement of the CLA in memory labilization/reconsolidation, we conducted the third set of pharmacological experiments. Lidocaine was administered either before or immediately after the first test session (TS1 - memory reactivation) and mice were retested 24 hours later. In our experimental conditions, lidocaine only impaired mice performance when administered immediately after memory reactivation. Conversely, CLA inactivation may not be essential for memory retrieval, as mice performance remained unaffected when the pharmacological treatment was administered before memory reactivation.

We conducted additional experiments to assess whether the involvement of the CLA in behavioral performance was specific to memory reactivation. For this purpose, lidocaine was administered to mice that did not undergo memory reactivation (NR group in Figure 4C) and tested 24 hours later. The results of this experiment suggest that the impairment in mice performance induced by lidocaine could not be attributed to a nonspecific effect of the pharmacological treatment, as lidocaine was unable to alter retention latencies without memory reactivation. Furthermore, the impairment could not be attributed to interference with long-lasting memory consolidation processes. To our knowledge, this study represents the initial demonstration elucidating the specificity of the CLA's engagement in memory reactivation and restabilization, offering novel perspectives on the contribution of this brain region to memory functions.

The study of memory reconsolidation may appear deceptively simple, but it is actually highly complex, with many aspects still remaining elusive and numerous drawbacks and obstacles to overcome. To evidence reconsolidation, memory must first be destabilized during a reactivation session, typically achieved by presenting the conditioned stimulus (CS) without the unconditioned stimulus (US), as described by Misanin et al. (89) and Nader et al. (42). Since no repetition of CS–US pairing was presented during the memory reactivation session, the subject is now exposed to different information, which could lead to the formation of a new memory (i.e., entering the dark compartment is not followed by the footshock). In the IA task, this would be observed as the animal entering the dark compartment increasingly faster, resulting in a shorter latency to step-through. This progressive reduction of the conditioned response due to repeated presentation of the CS in the absence of the US is known as memory extinction. Memory extinction can be modulated by interventional treatments: if this process is enhanced, the reduction of the conditioned response is faster, but if impaired, the animal is expected to behave without reducing the conditioned response. Thus, when a subject is exposed to a reactivation session, this reactivation triggers at least one of two memory processes: reconsolidation and/or extinction (90).

At this point, we might question whether administering a treatment after a reactivation session, leading to memory impairment evaluated 24 hours later, could be attributed to a failure in storage or to other processes such as memory retrieval or expression. The absence of a reversal of amnesia could support interpretations of a "storage deficit," akin to the deficit assumed to occur after consolidation blockade. Conversely, the reversibility of amnesia favors interpretations of "retrieval or expression deficits" (91). In our experimental conditions, employing a spontaneous recovery protocol to assess memory recovery, as described by Myers and Davis (90), lidocaine administered immediately after training not only affected mice performance tested 24 hours afterward but also 17 days later. Since there was no recovery of mice performance,

at least at the time points evaluated, our results suggest that the CLA is critical for memory re-storage/update, supporting the “storage deficit” interpretation. Furthermore, when treated mice were tested 2 hours after memory reactivation, they performed similarly regardless of the pharmacological treatment, suggesting that mice do not form an extinction memory. Altogether, our results point to a critical involvement of the CLA in the memory reconsolidation of an IA task.

3.4 Concluding remarks

To sum up, our study sheds light on the pivotal role of the CLA in memory processes, particularly in memory consolidation and reconsolidation. Through a combination of behavioral tasks and pharmacological interventions, we provided novel evidence suggesting that this often overlooked brain structure is crucial for the storage and updating of memories. Additionally, our findings reveal a correlation between neural activity in the CLA and memory-related processes, as evidenced by the upregulation of c-Fos expression following training in an IA task. Furthermore, our findings may hold translational significance for research into neurological pathologies and neurodegenerative diseases, as understanding the role of the CLA in memory processes could potentially offer new insights into the development of therapeutic interventions aimed at mitigating cognitive decline associated with these conditions. These insights not only contribute to a deeper understanding of the intricate mechanisms underlying memory but also underscore the significance of the CLA in memory-related cognitive functions.

4. Materials and methods

4.1 Experimental subjects

We used CF-1 male mice from our own breeding stock (age: 60–70 d; weight: 25–30 g). They were housed in groups of 5-10 mice per cage (cage size: 50 × 30 × 15 cm)

with food and water freely available, and were kept in a 12:12-hour light–dark cycle (lights on at 06.00 h) in a temperature regulated (23–25 ◦C) environment. We conducted the experiments between 08.00 and 14.00 h and carried them out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication N◦ 80–23/96) and local guidelines establish by CICUAL-FFyB (CUDAP # 0044975-2016). All efforts were made to minimize animal suffering and to reduce the number of animals used.

4.2 Stereotaxic surgery and intraclaustral injections

We anesthetized mice with isoflurane and secured them in a stereotaxic apparatus. The surgical procedure involved making an incision in the scalp and, to access the claustrum (CLA), we drilled a hole in the skull of each hemisphere at the following stereotaxic coordinates: AP +1.0 mm relative to bregma, L ±2.8 mm from the midsagittal suture. Then, we lowered a 30 G stainless-steel needle into each hemisphere in a 10 degree angle from the sagittal plane (-3.8 mm from the skull surface, Figure 1A; (49)) and bilaterally infused the claustrum with either a 2% lidocaine or saline solution (see Drugs section). Injections were driven by an automatic pump (MD-1020, Bioanalytical Systems, Inc.) at a rate of 0.52 uL/min through the needle attached to a 5 µl Hamilton syringe with PE-10 tubing. The volume of each infusion was 0.2 µl/hemisphere. We closed the incision with a stitch and the animals were returned to their home cages.

We determined the accuracy of the injections by histological examination of the needle position on an animal-by-animal basis. For this purpose, we dissected and fixed the animals' brains in 4% paraformaldehyde in buffer phosphate saline. Then, we cut them into 200 nm coronal sections with a vibratome. The deepest position of the needle was superimposed on serial coronal maps (Franklin & Paxinos, 1997). Animals were excluded from the statistical analysis if the infusions had caused excessive damage to the cerebral tissue or if the needle trajectory extended outside of the claustrum.

4.3 Drugs

We bilaterally administered a solution of 2% lidocaine (Lido; Laboratorios Veinfar I.C.S.A.. Bs. As) or saline (Veh). We determined this Lido dose based on previous work from McIntyre and collaborators (50). All other agents were of analytical grade and obtained from local commercial sources.

4.4 Behavioral Procedures

4.4.1 Inhibitory Avoidance task

We studied inhibitory avoidance (IA) behavior in a one-trial learning, step-through type situation (51,52), which utilizes the natural aversion of mice for exposed and illuminated environments. The apparatus consists of a dark compartment (20 x 20 x 15 cm) with a stainless-steel grid floor and a small (5 x 5 cm) illuminated, elevated platform attached to its front center. During training, we placed each mouse on the platform and, as it stepped into the dark compartment, it received a foot-shock (0.3 mA, 5 Hz, 1 s). Forty-eight hours later, we placed each mouse on the platform again and the step-through latency was recorded (testing session, TS). If a mouse failed to cross within 300 s, the retention test was terminated and the mouse was assigned a ceiling score of 300. The mice were naïve to the dark compartment before the learning trial. When indicated, we conducted subsequent testing sessions at the designated time points for each experiment.

4.4.2 Nose-poke habituation task

We also studied the nose-poke habituation task (HT) in a one-trial learning situation on a hole-board. The apparatus (Ugo Basile Mod. 6650, Comerio, Italy), made of a gray Perspex panel (40 x 40 x 22 cm), embodies 16 flush mounted tubes of 3 cm of diameter. Each tube has an infrared emitter and a diametrically opposed receiver connected to an automatic counter to register the number of nose-pokes into the holes. The training and testing trials were 24 h apart. During both trials, we placed each mouse

at the center of the apparatus and the number of nose pokes was automatically registered for 5 min. From one mouse to the next, we carefully cleaned the hole-board with ethanol 70% to avoid olfactory cues.

4.5 Brain fixation and sectioning for Immunohistochemistry

Ninety minutes after the IA training, we deeply anesthetized animals with ketamine and xylazine, and fixed their tissue by intracardiac perfusion of ~10 mL of ice-cold 0.1% heparin phosphate buffered saline (PBS), followed by ~25 mL of ice-cold 4% paraformaldehyde (PFA) in PBS. We then dissected their brains, which were post-fixed overnight in the same fixing solution. After 24 h, we first cryopreserved the tissue in a 15% sucrose solution in PBS at 4 °C for 24 h, then in a 30% sucrose solution in PBS for another 24 h and maintained it in a fresh 30% sucrose solution until sectioned. We embedded in Tissue-Tek (Sakura) and cryotome sectioned the brains at a thickness of 40 µm. Sections including CLA (-0.50 to 1.50 AP from bregma), CA1 area of the hippocampus (-2.50 AP to -1.50 from bregma) and motor cortices (0.00 to 1.50 AP from bregma; M1/2) were collected (49).

4.6 Immunohistochemistry (IHC)

We washed with 0.1% Triton in PBS and blocked the selected brain sections with 1% donkey serum (D9663, Sigma-Aldrich) in the wash solution for 60 m at room temperature (RT). Then, incubated the slices overnight at RT with 1:500 rabbit α c-Fos (ABE457, Merck Millipore) and 1:3000 mouse α parvalbumin (PV 235, Swant) antibodies. After 24 h, we washed them with 0.1% Triton in PBS, followed by incubation with 1:1000 Biotin donkey α mouse (715-065-151, Jackson ImmunoResearch). After 120 min, we again washed the sections with 0.1% Triton in PBS, then with PBS and lastly with phosphate buffer (PB), before incubating them with 1:200 donkey α rabbit Alexa 488 (711-545-152, Jackson ImmunoResearch) and 1:200 Streptavidin Cy3 (016-160-064, Jackson ImmunoResearch) in PB. After 120 min, brain sections were washed with PB,

dried at RT, re-hydrated in PB and mounted in gelatinized slides (gelatine type a g8-500, Fisher Chemical) using Mowiol as mounting medium (81381, Sigma-Aldrich).

4.7 Acquisition and processing of images

We acquired images using a LSM900 Zeiss confocal microscope and a 20x objective (Carl Zeiss, Plan Apochromat, NA 0.8). Z-stacks spanning the entire thickness of each slice were obtained with 512 × 512 pixel resolution, parvalbumin (PV) and c-Fos acquisition channels were separated and images were processed with ImageJ. Briefly, we delineated a CLA-mask using the signal present in the PV channel. Then, the signal from c-Fos channel was collapsed to a maximum intensity projection and the CLA-mask superimposed. We normalized the amount of c-Fos positive cells to the mask area for each image. CA1 and M1/2 masks were performed based on the atlas of Franklin and Paxinos (49).

4.8 Data analyses

We show data representing latency to step-through, cumulative nose pokes and relative density of c-Fos positive cells in plots to depict summary statistics, as well as the distribution of individual values. For normally distributed data, we depict the mean and standard error as summary statistics, otherwise we show the median and interquartile range (IQR). The summary statistics and final amount of mice included in the analysis of each experiment is specified in its respective Figure legend. We performed data analysis with R (version 4.2.2). To account for data dependency (as multiple observations were drawn from each animal), generalized linear mixed models (GLMMs) were performed using the *glmmTMB* package (version 1.1.6)(53). For behavioral data, we fitted a GLMM for each variable of interest with Group (Veh and Lido) and Trial (TR, TS1, TS2 and TS3 in Figures) as fixed effects, and mouse ID (Subject) for random effects. We added the Minute fixed effect (1 through 5 or 15) for HT experiments. For IHC data, we fitted a GLMM for c-Fos density in each image of CLA, CA1 or M1/2 with Group (Na and TR) as

fixed effect and mouse ID (Subject) for random effect (ANOVA of GLMMs are displayed in Suppl. Table 1). The GLMM corresponding to the latency to step-through (IA experiments) was fitted with a Gamma family distribution and a log link function, while the LMM for cumulative nose pokes (HT experiments) and IHC experiments were fitted with a Gaussian family distribution and an identity link function. The goodness-of-fit for each model was analyzed using the *DHARMa* package (version 0.4.6). Visual inspection of residual plots did not reveal any concerning deviations from normality or homoscedasticity. When applicable, we performed post-hoc comparisons using the *emmeans* package (version 1.8.5)(54) and adjusted all reported p-values for multiple comparisons with the Šidák correction method. In all cases, p values under 0.05 were considered significant. When indicated, $*$ signifies $p < 0.05$; $**$, $p < 0.01$; and $***$, $p < 0.001$.

4.9 Experimental design and groups

We performed a total of eleven experiments. Except for experiments 1 and 5-7, we trained animals in the IA task and evaluated their behavioral performance 48 h later as the latency to step-through and into the dark compartment. In experiment 1, we deeply anesthetized animals and fixed their brains 90 min after training. In experiments 5-7, we trained mice in the HT and evaluated their behavioral performance either 2 or 24 hours later as the accumulated nose pokes in each trial.

4.9.1 Experiments 1, 2, 3, 4, 13 and 14: The claustrum in the acquisition and consolidation of an associative memory

The first set of experiments was performed in order to evaluate neural activity elicited by the IA training trial within the CLA, CA1 and M1/2 (experiment 1, Figure 1) and the effect of the inhibition of neuronal transmission in the CLA during the acquisition and consolidation of this associative aversive memory (experiments 2-4, Figure 2).

In experiment 1, we trained a group of 4 mice in the IA task and, 90 minutes later, we fixed their brains for evaluation of the immediate early gene c-Fos' positive cells through IHC, as a proxy for neural activity. This time point was selected based on the peak in the time course of c-Fos expression observed in the rat visual cortex following visual stimulation, as reported by Chaudhuri and colleagues (55). Together with this group, we performed the same protocol in 4 behaviorally naïve animals, as a control group for neural activation. Between 2 and 14 images were analyzed per mouse in each experimental group.

In the second experiment of this series our goal was to test the role of the CLA in the acquisition of an IA memory. We administered bilateral infusions of either Veh or Lido into the CLA of two groups of mice 1 h before the IA training trial (8 and 9 animals, respectively) and 2 h later tested their latency to step-through, in order to evaluate short-term memory. We determined this time point for Lido infusion in the CLA by maximizing the time window between isoflurane administration and the behavioral trial, taking into account the elimination half-life of Lido (56) and that isoflurane-administered animals do not behave significantly different from control mice 60 m after anesthesia (data not shown).

In experiment 3, we bilaterally administered either Veh or Lido in the CLA of two groups of mice immediately after the IA training trial (8 and 9 animals, respectively), in order to specifically evaluate its role in memory stabilization. The last experiment of this series was very similar, but aimed at evaluating if the effect of Lido on the animals' behavioral performance was time-specific. Therefore, we infused 8 animals with Veh and 7 with Lido 180 minutes after IA training trial. These last two experiments were repeated using female mice (experiment 13 and 14, respectively). In experiment 13 we bilaterally administered either Veh or Lido in the CLA of two groups of female mice immediately after the IA training trial (7 and 6 animals, respectively), while in experiment 14 we infused 10 animals with Veh and 10 with Lido 180 minutes after IA training trial.

4.9.2 Experiments 5, 6 and 7: The claustrum in the acquisition and consolidation of an habituation memory

In this second set of experiments, depicted in Figure 3, we aimed at testing whether the CLA is relevant for the stabilization of an nonassociative memory. In experiment 5, we aimed to test whether synaptic transmission in the CLA is necessary for the consolidation of a HT memory by bilaterally administering either Veh or Lido in the CLA immediately after a 5 min HT training trial (5 and 6 animals for each group). As in the first set of experiments, in experiment 6 we evaluated the time-specificity of the Lido effect on memory by administering two additional experimental groups with either Veh or Lido 3 h after the training trial (7 and 11 mice, respectively).

In experiment 7, we evaluated the role of the CLA in the acquisition of a HT memory by bilateral infusion of either Veh or Lido in the CLA of another set of experimental groups 1 h before a 15 min HT training trial (7 mice each).

4.9.3 Experiments 8, 9, 10 and 11: The claustrum in the retrieval and reconsolidation of an associative memory

With this third set of experiments we sought to elucidate if the CLA's synaptic activity is necessary for the retrieval and labilization/reconsolidation processes following IA memory reactivation (Figure 4).

First, in experiment 8, our goal was to elucidate the role of CLA in the retrieval of an IA memory. Then, we infused two experimental groups with either Veh or Lido 1 h prior to TS1 and then evaluated 24h later (TS2; 10 and 7 animals, respectively).

Next, we performed experiment 9 in order to better understand the role of CLA in the labilization/reconsolidation processes. We trained two groups of mice in the IA task and tested them 48 h later, in a trial entailing memory reactivation (TS1; (57)). Immediately after TS1, 7 mice were infused with Veh and 12 with Lido, within the CLA. We evaluated their behavioral performance 24 h (TS2) and 15 d (TS3) later.

In order to investigate if the effect evaluated in experiment 9 is time-specific and dependent on memory reactivation/labilization, we performed experiment 10. The experimental protocol was similar to experiment 9, except we skipped the TS1 trial, avoiding memory reactivation (NR groups). We administered mice in the CLA with either Veh (8 animals) or Lido (6 animals) 48 h after the training trial and evaluated their behavioral performance 24 h later.

Lastly, we wanted to test whether the inhibition of the CLA's neural activity during memory restabilization by Lido administration could facilitate the formation of a novel extinction memory. Therefore, we performed experiment 11 similarly as experiment 9, with the exception of re-testing the animals (TS2) 2 h after memory reactivation, in order to evaluate the formation of a short-term memory. Therefore, we administered either Veh or Lido bilaterally in the CLA of another set of experimental groups, as follows: 9 animals were administered with Veh immediately after TS1, 10 animals were injected with Lido immediately after TS1 and 9 extra animals were also infused with Lido in the CLA 48h after training, while skipping TS1 trial (NR-Lido experimental group).

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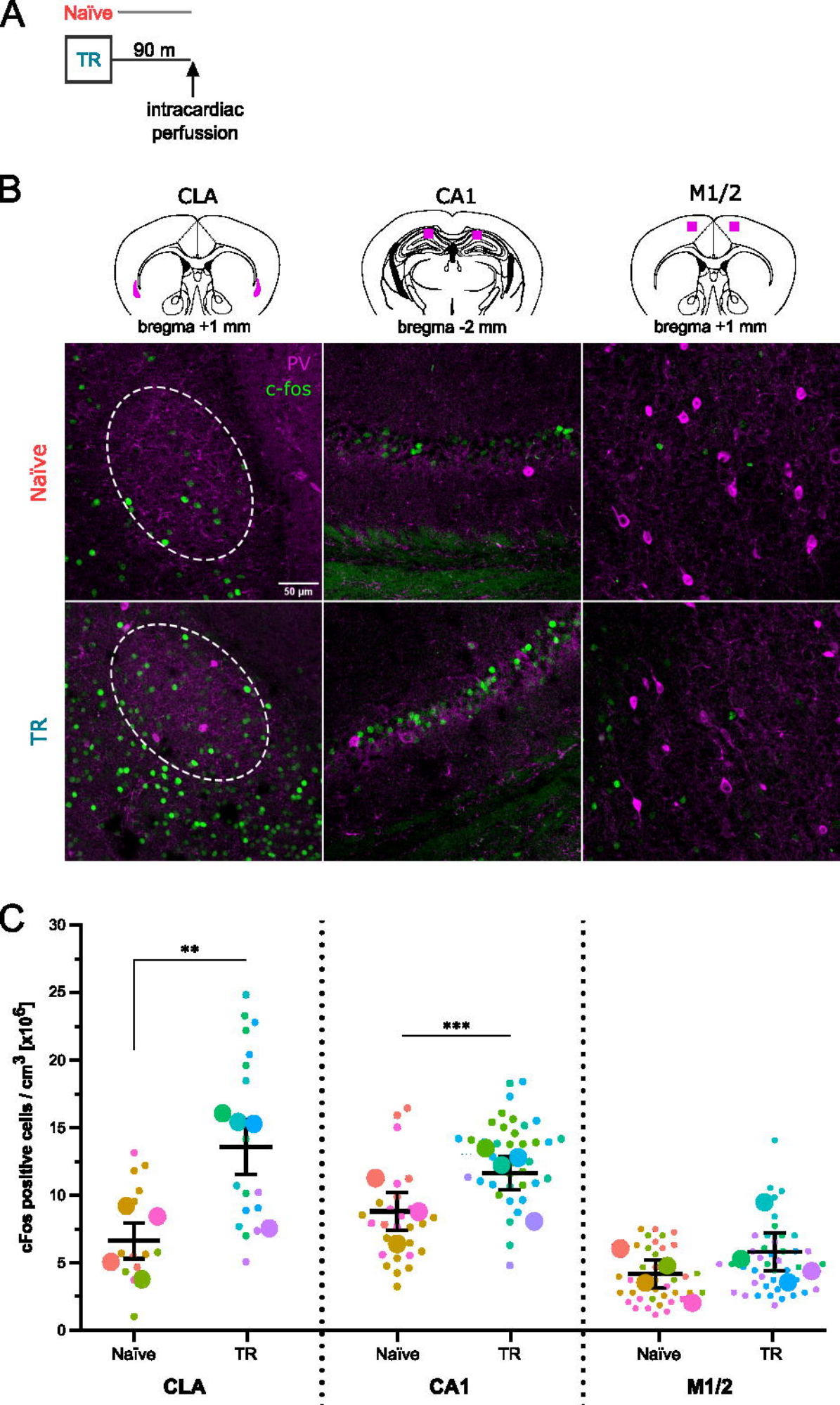
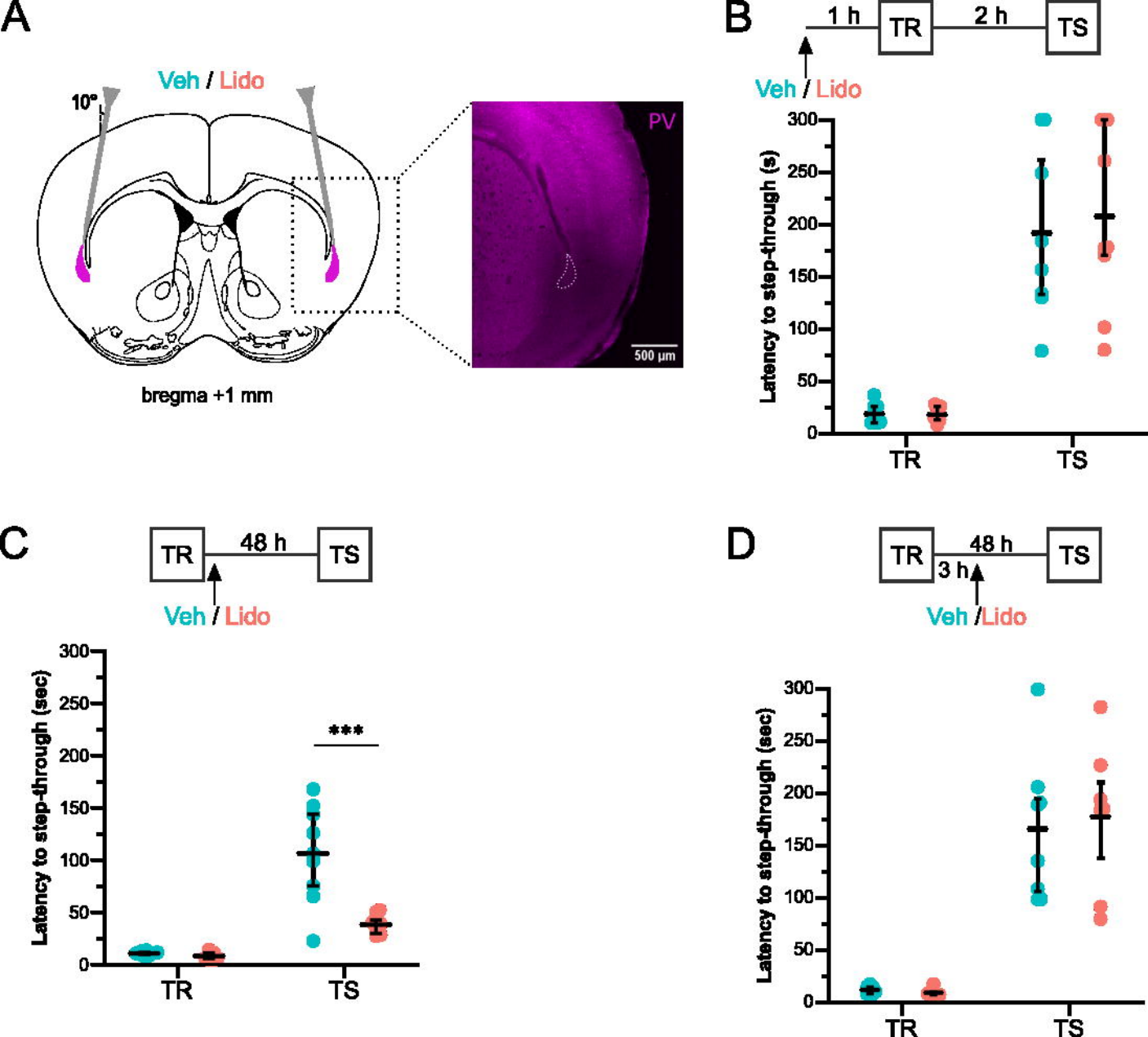
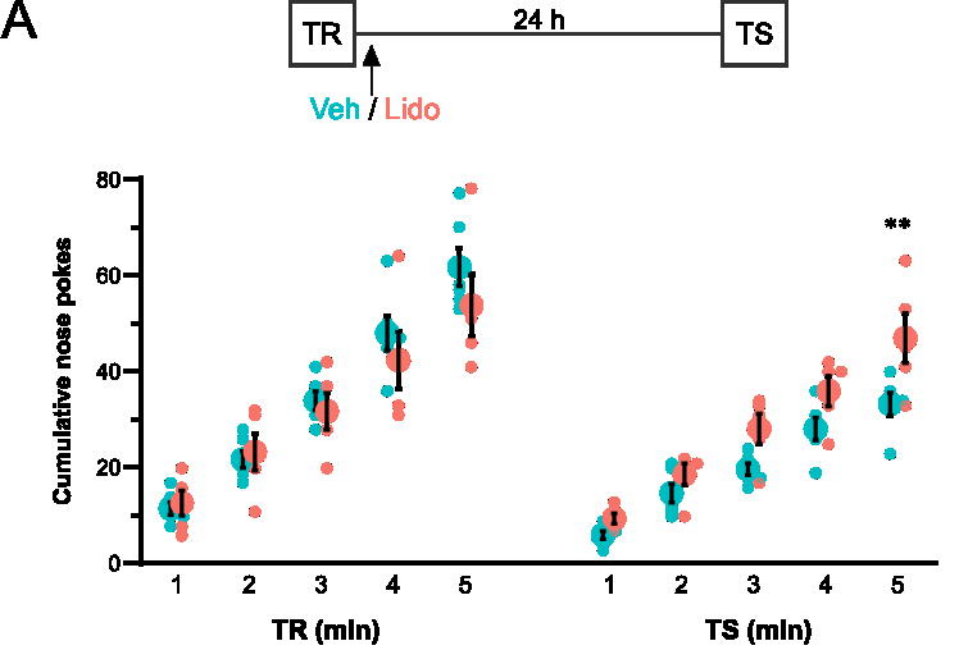
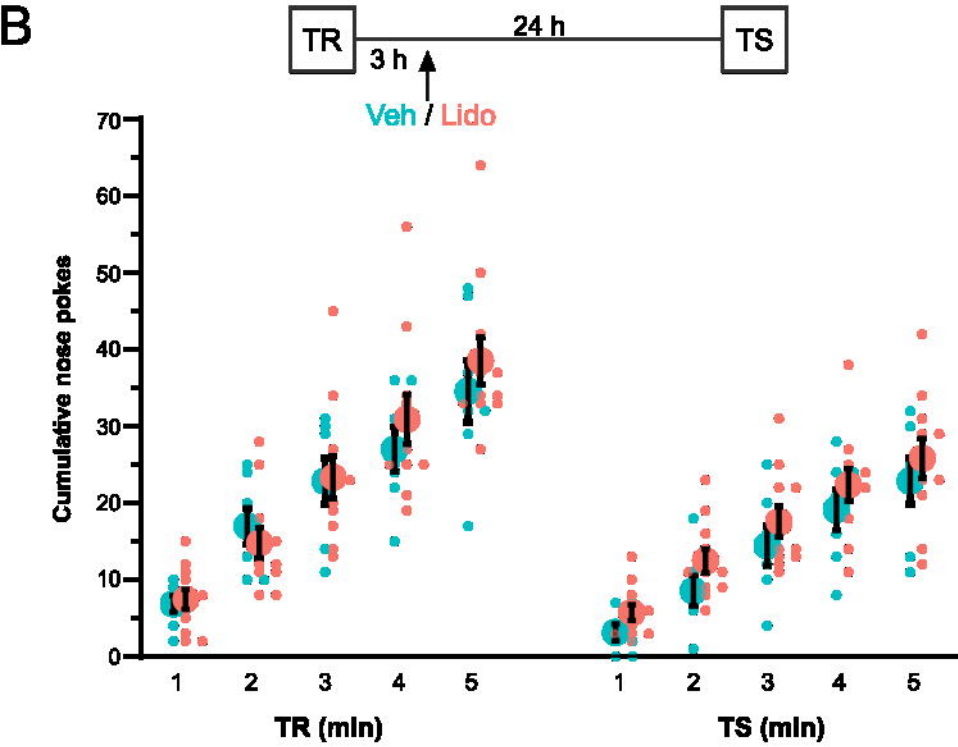

A
Naïve
TR
90 m
intracardiac perfussion
B
CLA
CA1
M1/2
bregma +1 mm
bregma -2 mm
bregma +1 mm
PV
c-fos
50 µm
Naïve
TR
C
cFos positive cells / cm3 [x10^6]
**
***
Naïve
TR
Naïve
TR
Naïve
TR
CLA
CA1
M1/2


A
Veh / Lido
10°
PV
500 µm
bregma +1 mm
B
1 h
TR
2 h
TS
Veh / Lido
Latency to step-through (s)
300
250
200
150
100
50
0
TR
TS
C
TR
48 h
TS
Veh / Lido
Latency to step-through (sec)
300
250
200
150
100
50
0
***
TR
TS
D
TR
48 h
TS
3 h
Veh /Lido
Latency to step-through (sec)
300
250
200
150
100
50
0
TR
TS

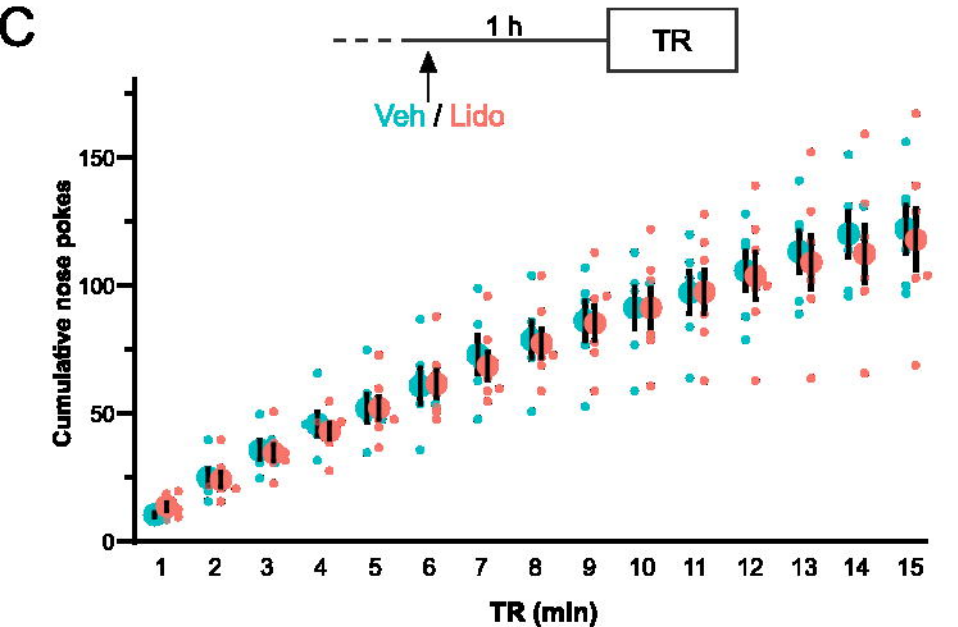
A

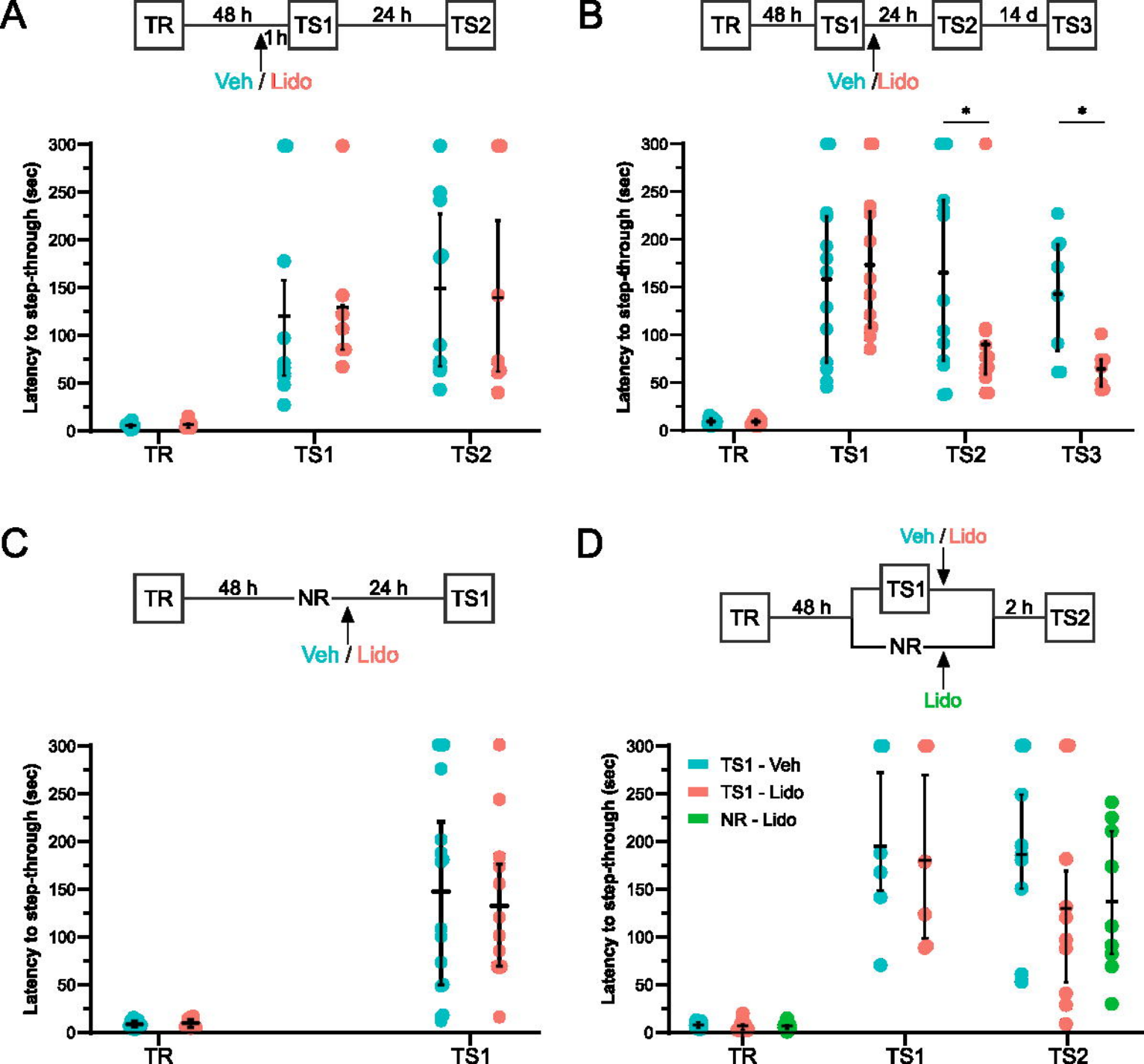

TR
24 h
TS
Veh / Lido
Cumulative nose pokes
80
60
40
20
0
**
1
2
3
4
5
TR (min)
TS (min)

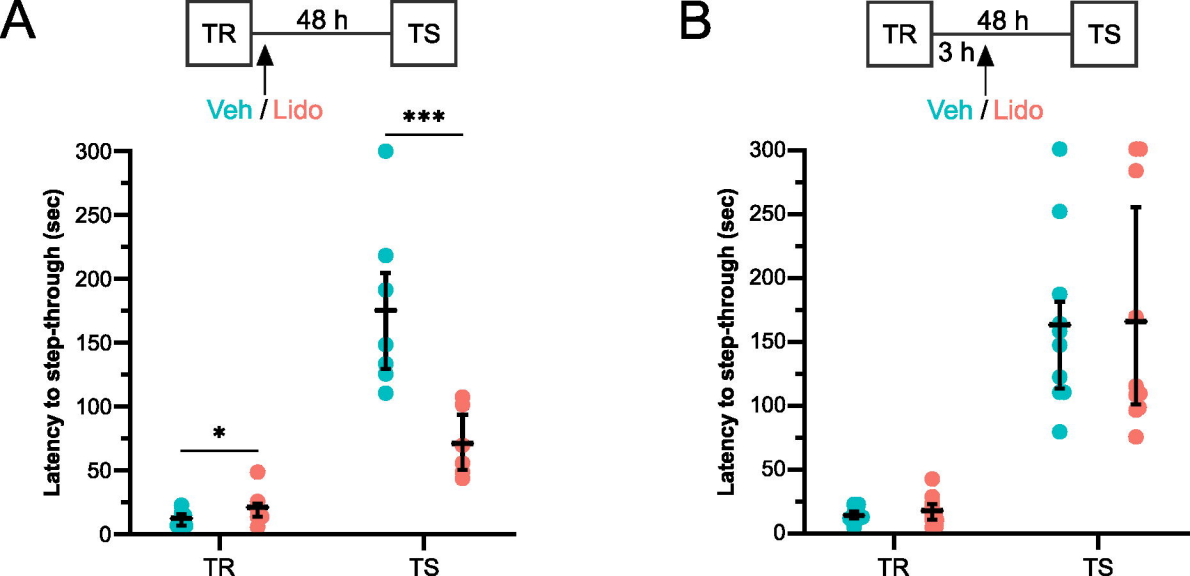
B


TR
24 h
TS
3 h
Veh / Lido
Cumulative nose pokes
70
60
50
40
30
20
10
0
1
2
3
4
5
TR (min)
TS (min)

C


1 h
TR
Veh / Lido
Cumulative nose pokes
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
TR (min)


A
TR
48 h
TS1
24 h
TS2
1 h
Veh / Lido
Latency to step-through (sec)
300
250
200
150
100
50
0
TR
TS1
TS2
B
TR
48 h
TS1
24 h
TS2
14 d
TS3
Veh / Lido
*
*
Latency to step-through (sec)
300
250
200
150
100
50
0
TR
TS1
TS2
TS3
C
TR
48 h
NR
24 h
TS1
Veh / Lido
Latency to step-through (sec)
300
250
200
150
100
50
0
TR
TS1
D
Veh / Lido
TS1
TR
48 h
2 h
TS2
NR
Lido
TS1 - Veh
TS1 - Lido
NR - Lido
Latency to step-through (sec)
300
250
200
150
100
50
0
TR
TS1
TS2


A
TR
48 h
TS
Veh / Lido
***
*
Latency to step-through (sec)
300
250
200
150
100
50
0
TR
TS
B
TR
48 h
3 h
TS
Veh / Lido
Latency to step-through (sec)
300
250
200
150
100
50
0
TR
TS