Research

Maintenance of conditioned place avoidance induced by gastric malaise requires NMDA activity within the ventral hippocampus

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It has been reported that during chemotherapy treatment, some patients can experience nausea before pharmacological administration, suggesting that contextual stimuli are associated with the nauseating effects. There are attempts to reproduce with animal models the conditions under which this phenomenon is observed to provide a useful paradigm for studying contextual aversion learning and the brain structures involved. This manuscript assessed the hippocampus involvement in acquiring and maintaining long-term conditioned place avoidance (CPA) induced by a gastric malaise-inducing agent, LiCl. Our results demonstrate that a reliable induction of CPA is possible after one acquisition trial. However, CPA establishment requires a 20-min confinement in the compartment associated with LiCl administration. Interestingly, both hippocampal regions seem to be necessary for CPA establishment; nonetheless, inactivation of the ventral hippocampus results in a reversion of avoidance and turns it into preference. Moreover, we demonstrate that activation of dorsal/ventral hippocampal NMDA receptors after CS–US association is required for long-term CPA memory maintenance.

After repeated chemotherapy cycles for cancer, some patients developed unpleasant side effects like strong nausea. In those cases, patients develop anticipatory nausea responses before the chemotherapy drug administration (Symonds and Hall 2012; Rodríguez 2013). This phenomenon suggests that the administration of toxic drugs is associated with environmental cues. Therefore, it is necessary to understand, with animal models, the conditions under which this phenomenon is produced to improve the understanding of the maintenance of contextual aversion learning induced by visceral consequences and the brain structures involved.

In general, contextual aversion learning has been widely analyzed through behavioral paradigms such as contextual fear conditioning, which involves both spatial and aversive elements (Staib et al. 2018; Shallie and Mabandla 2020). Likewise, place conditioning paradigm is a behavioral model to study the rewarding and aversive effects of drugs through the association of a particular context with drug treatment (Prus et al. 2009). This paradigm has been widely used to study the motivational effects of abused drugs (Cunningham et al. 2006). In this regard, conditioned place avoidance (CPA) is a protocol designed to explore the biological mechanisms underlying aversive responses induced by substance dependence withdrawal (Song et al. 2017). However, the association of a unique context with interoceptive aversive cues has not yet been extensively studied.

In the CPA, different types of stimuli may serve as an aversive stimulus. For example, electric shocks, formalin injections and intraperitoneal lithium chloride (LiCl) injections can be used as an unconditioned stimulus (Rinaman et al. 2009; Jiang et al. 2014). In the latter case, LiCl is a substance capable of inducing gastric malaise and nausea, so it is more frequently used in conditioned taste aversion experiments. Although the LiCl administration in CPA protocols is scarcely used, the mechanisms by which lithium chloride induces a place avoidance and the brain structures involved are not fully described.

Most of the literature concerning contextual learning and memory has focused on the hippocampus; this brain structure became relevant in the field since the pioneering work on the H. M. case by Scoville and Milner (1957). Since then, a considerable amount of evidence supports the crucial role of the hippocampus in episodic memories in primates, rats and mice (Squire 1992). Ramón y Cajal and Lorente de Nó (revised in Fanselow and Dong 2010) described the cytoarchitectonic distribution of the hippocampus, and the structural differences across the longitudinal axis, distinguishing a dorsal and a ventral portion. Later studies based on genomic and connectivity analyses (Witter and Amaral 2004; Dong et al. 2009) strengthened the idea that dorsal and ventral portions are functionally different, suggesting that the hippocampus does not act as a unitary structure (Moser and Moser 1998). Moreover, a vast amount of behavioral data has concluded that the dorsal hippocampus is primarily related to spatial or contextual information processing. The ventral portion is considered a region that contributes to emotional content codification, especially in stressful and anxiety-related situations (Fanselow and Dong 2010).

Notwithstanding the well-established dual hippocampal function, most research articles consider and evaluate only the dorsal or ventral portion in their experiments. To our knowledge, few studies evaluated the participation of both portions of the hippocampus in the same task. Therefore, the purpose of the following experiments was to determine the role of both dorsal and ventral hippocampal regions in the association of a context with gastric malaise in a CPA task that might ascertain the neurobiological substrates involved in anticipatory nausea associated with

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contextual stimuli. Our first approximation consisted of a general inactivation through an intracerebral infusion of a $GABA_A$ and $GABA_B$ agonist cocktail during CPA acquisition. Additionally, it is well known that the hippocampus is a region characterized for having a profuse expression of NMDA receptors (Dingledine 1983). Thus, we further assessed NMDA receptors' role in acquiring and maintaining CPA through memory consolidation in each hippocampal region. Figure 1 represents a summary of the experimental design.

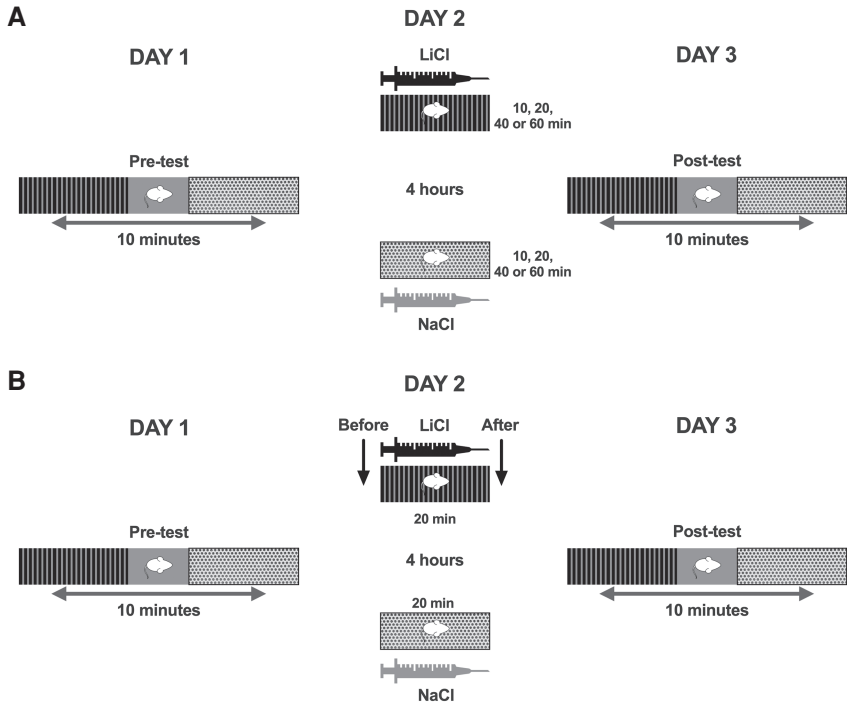

Figure 1. Protocol schemes of Conditioned Place Avoidance protocol. (*A*) On the pretest session, the rat was placed at the middle compartment and freely explored the chambers over a period of 10 min period. On day 2, during the conditioning session, animals were injected i.p. with 0.4M LiCl (7.5 mL/kg) and confined to the black chamber for 10, 20, 40, or 60 min; 4 h later, rats were injected i.p, with 0.15 M NaCl (7.5 mL/kg) and confined to the white chamber for 10, 20, 40, or 60 min, respectively. On the post-test session, the rat was placed in the middle compartment and freely explored the chambers over a period of 10 min. (*B*) In a similar CPA protocol, during the infusion session on day 2, a volume of 0.5 µL (0.25 µL/min) of elected drug was bilaterally injected in the dorsal or ventral hippocampus. Infusion times were 15 min before the CS–US association or immediately after the CS–US association. Injected drugs were saline solution 0.9% (w/v), a $GABA_A$ and $GABA_B$ agonist cocktail (0.1 mM Muscimol and 1 mM Baclofen), or 10 µg/µL DL-2-amino-5-phosphonovaleric acid (APV). Accordingly to our results, 20-min confinement induces a robust CPA. Therefore, all pharmacological manipulations were conducted in 20-min association protocol.

Results

Confinement time

Figure 2 shows that the induction of CPA by i.p. 0.4 M LiCl in rats is possible with one acquisition trial only under specific temporal conditions of confinement. One-way ANOVA showed significant differences among means scores ($F_{(3,24)} = 4.184$, $P = 0.0162$). Fisher's post hoc test displayed differences in CPA scores between the rats that were confined for 20 min compared with 10 ($P = 0.0062$), 40 ($P = 0.0275$) or 60 min ($P = 0.0083$) confinement. No differences were observed with one-sample *t*-test in 10-, 40-, or 60-min confinement compared with the hypothetical value of 0. Importantly, the rats confined for 20 min were different from the hypothetical value of 0 ($t = 5.405$, $P = 0.001$), indicating a clear avoidance behavior. Thus, 20 min of confinement is required to acquire a reliable CPA through LiCl administration.

After determining the temporal conditions for CPA establishment, we evaluated whether CPA formation resulted from the association between the context and abdominal irritation by hypertonic solution or emesis-eliciting information induced by lithium (Fig. 2B). Therefore, we analyzed the CPA performance in four groups that received the following solutions in the preferred compartment: 0.15 M isotonic NaCl solution (7.5 mL/kg, $n = 8$), 0.4 M hypertonic NaCl (7.5 mL/kg, $n = 8$), 0.15 M isotonic LiCl solution (7.5 mL/kg, $n = 7$), or 0.4 M hypertonic LiCl solution (7.5 mL/kg). One-way ANOVA showed significant differences among means scores ($F_{(3,27)} = 5.881$, $P = 0.0032$). Fisher's post hoc test displayed differences in CPA scores between the group that was injected i.p. with 0.4 M LiCl when compared with 0.15 M NaCl ($P = 0.0003$), 0.4 M NaCl ($P = 0.0095$) or 0.15 M LiCl ($P = 0.048$) groups. One-sample *t*-test reported that 0.15 M NaCl, 0.4 M NaCl, and 0.15 M LiCl groups spent a similar amount of time compared with the pretest (hypothetical value of 0). Our results suggest that CPA establishment is possible after 20 min of confinement following 0.4 M LiCl administration. Noteworthy, abdominal pain is not sufficient to promote CPA since the administration of a hypertonic NaCl solution fails to induce CPA even after a writhing effect. Moreover, CPA establishment requires a hypertonic LiCl to induce a reliable behavior suggesting that high toxicity or emesis-eliciting information is required to induce this kind of CPA.

We then examined the involvement of ventral and dorsal regions in CPA formation (Fig. 3). Statistical analysis revealed a drug main effect ($F_{(1,23)} = 18.17$, $P = 0.0003$). Neither region ($F_{(1,23)} = 1.359$, $P = 0.2557$) nor interaction effect was observed ($F_{(1,23)} = 4.049$, $P = 0.056$). Fisher's post hoc test displayed differences in CPA scores between Ventral-SS and Ventral M/B groups ($P = 0.0001$), and between Dorsal-M/B and Ventral M/B groups ($P = 0.0343$). One-sample *t*-tests reported that Dorsal-SS ($t = 3.897$, $P = 0.0176$), Ventral-SS ($t = 2.889$, $P = 0.0202$) and Ventral-M/B ($t = 3.133$, $P = 0.0259$) groups were different from the hypothetical value of 0, whereas Dorsal-M/B group was similar; suggesting a clear CPA for Dorsal-SS and Ventral-SS groups and a preference for the black compartment in the Ventral-M/B group. To elucidate whether the administration of M/B into the ventral hippocampus could induce a conditioned place preference, we performed a control group that received an i.p. administration of LiCl and confined to the black compartment; 4 h later, rats were injected with M/B into the ventral hippocampus, received an i.p. administration of NaCl and confined to the white compartment. This group acquired a reliable CPA for the black compartment and did not promote a preference for the white compartment (data not shown). Therefore, inhibition of the dorsal and ventral hippocampus impaired CPA memory. However, inhibition of the ventral region prevents CPA establishment and promotes an increase in the time spent in the black compartment.

NMDA receptor blockade experiments indicate significant differences in CPA scores (Fig. 4). Two-way ANOVA was used to

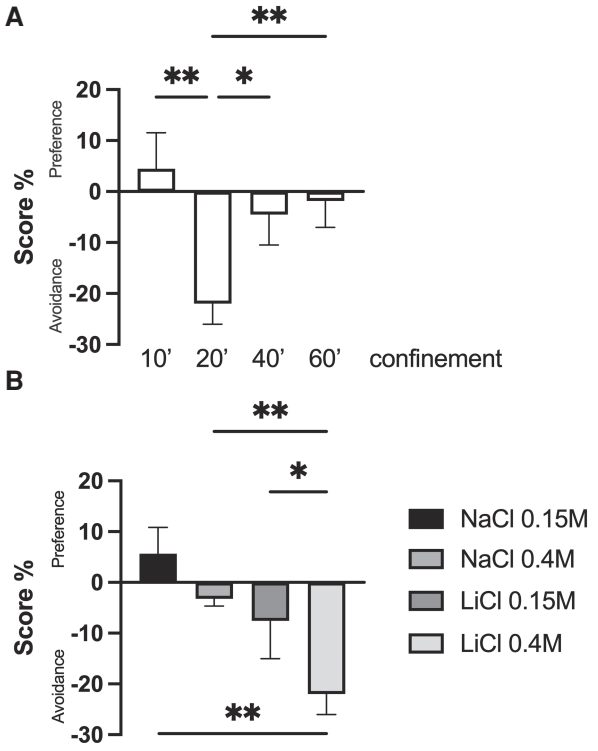

Figure 2. Conditioned place avoidance establishment. (*A*) A specific temporal condition of confinement is required for CPA establishment. (*B*) Administration of isotonic NaCl, hypertonic NaCl or isotonic LiCl fails to induce CPA. (*) $P < 0.05$, (**) $P < 0.01$ when compared with 20-min confinement or 0.4 M LiCl. Data are expressed as the mean of the score percentage ± SEM.

examine the effect of drug and region on CPA scores. When APV was administered before CS–US association, no effects were observed in Region ($F_{(1,27)} = 0.1797$, $P = 0.6751$), Drug ($F_{(1,27)} = 0.042$, $P = 0.8390$), or Drug × Region interaction ($F_{(1,27)} = 0.2516$, $P = 0.6202$). One-sample *t*-tests reported that Dorsal-SS ($t = 3.897$, $P = 0.0176$), Ventral-SS ($t = 2.889$, $P = 0.0202$), Dorsal-APV ($t = 2.991$, $P = 0.0202$), and Ventral-APV ($t = 4.252$, $P = 0.0038$) were different from a hypothetical value of 0 indicating a clear aversion in all groups. Our results suggest that administration of APV within ventral or dorsal hippocampal regions before acquisition spares CPA formation.

However, when the infusions of APV were after the CS–US pairing, we observed significant different scores ($F_{(1,26)} = 9.176$, $P = 0.0055$), whereas no region ($F_{(1,26)} = 0.2163$, $P = 0.6457$) or a interaction effect was observed ($F_{(1,26)} = 0.2958$, $P = 0.5911$). Post hoc analysis revealed differences between Ventral-SS and Ventral-APV ($P = 0.0189$). We observed a tendency for a diminished CPA score between Dorsal-SS and Dorsal APV ($P = 0.0878$). One-sample *t*-test showed that Dorsal-SS ($t = 2.665$, $P = 0.0323$) and Ventral-SS ($t = 2.747$, $P = 0.0252$) groups were different in comparison with a hypothetical value of 0, indicating avoidance behavior; scores in Dorsal-APV or Ventral-APV were similar to the hypothetical value of 0. Briefly, our results indicate that blockade of NMDA receptors after CS–US association impairs memory consolidation, suggesting that NMDA receptors' activity is necessary for CPA long-term memory consolidation and further memory maintenance.

Discussion

Considering the previous results, we demonstrated that a reliable CPA induced by LiCl is possible, providing a useful paradigm for studying anticipatory nausea observed in patients undergoing chemotherapy. We observed a differential involvement of hippocampus: While inactivation of the dorsal portion seems to block the establishment of conditioned place avoidance, inhibition of ventral hippocampus results in a reversion of avoidance and turn it into preference. Also, we elucidated the participation of hippocampal glutamatergic NMDA receptors during the consolidation stage of this task.

In the first place, CPA protocols for assessing learning and memory processes generally use the application of subcutaneous/intrapalmar injections of formalin, electric shocks, or intraperitoneal administrations of lithium chloride (LiCl) as aversive stimuli (Gao et al. 2004; Rinaman et al. 2009; Jiang et al. 2014). When administering LiCl, CPA protocols are often designed with repeated conditioning trials and several injections (Mallet and Beninger 1998; Ossenkopp et al. 2012; Cloutier et al. 2018). In aversion learning, it is known that particular cues and consequences are more readily associated than others according to specific characteristics such as the sensory modality (Garcia and Koelling 1966; Miller and Domjan 1981). Thus, LiCl administration is frequently used in paradigms of taste learning in which the conditioned stimulus is edible and is associated with a gastric malaise-inducing agent (Rabin and Hunt 1983, 1992). For example, conditioned taste aversion (CTA) experiments are well known to require one conditioning trial to have an evident and long-lasting aversion to the conditioned stimulus. These aversions could last days or weeks, depending on the stimulus' intensity (Garcia et al. 1955).

In our experiments, we induced a conditioned place avoidance with only one conditioning session and a single administration of hypertonic lithium chloride. Nevertheless, we observed this phenomenon under specific temporal conditions of confinement. We induced CPA by administering i.p. 0.4 M LiCl based on previous literature that found this concentration strongly aversive. These doses generate notable behavioral manifestations of gastric malaise (e.g., abdominal writhing effect, lying-on belly postures and diarrhea) at the moment of injection (Nachman and Ashe 1973; Bernstein et al. 1992).

Likewise, when standardizing this protocol, we considered different confinement durations, with the 20-min period being the only effective to induce CPA. The duration of LiCl effects may explain this phenomenon; it is known that most of the concentrations of this drug exert a maximum influence between 15 and 30 minutes after an intraperitoneal administration (Syme

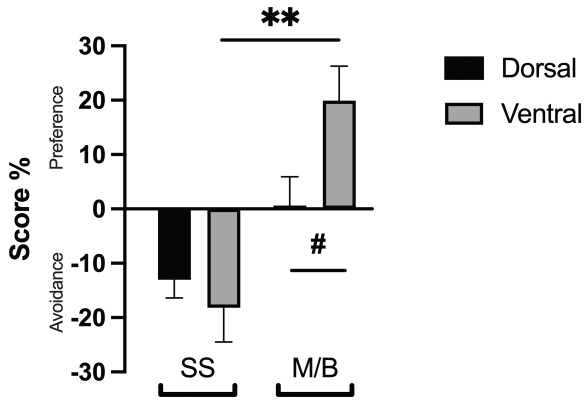

Figure 3. Pharmacological blockade of the ventral hippocampal regions disrupts CPA formation. Evaluation of memory retrieval indicates that administration of $GABA_A$ and $GABA_B$ agonist cocktail (0.1 mM Muscimol and 1 mM Baclofen) impairs CPA memory formation. (**) $P < 0.01$ compared with SS treatment, (#) $P < 0.05$ between dorsal and ventral regions comparison. Data are expressed as the mean of the score percentage ± SEM.

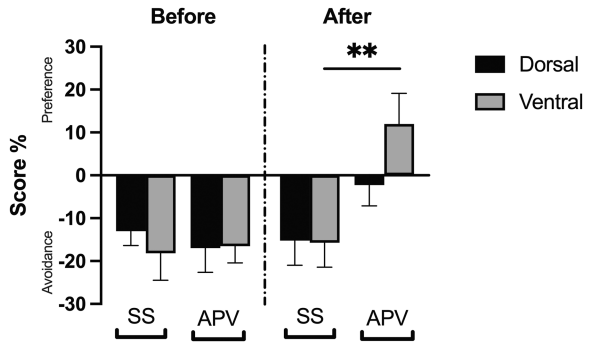

Figure 4. Pharmacological blockade of NMDA receptors within the ventral hippocampal region disrupts CPA memory maintenance. Evaluation of CPA memory retrieval indicates that administration of APV impairs CPA memory maintenance only if administered after CS–US association; (**) $P < 0.01$ compared with SS treatment in the ventral region. Data are expressed as the mean of the score percentage ± SEM.

and Syme 1973; Johnson 1976; Tomasiewicz et al. 2006). In this way, periods of confinement <15 min would fail to establish conditioned place avoidance since the pinnacle of lithium effects has not already been reached, so it is likely that the malaise-related symptoms are not restricted to the compartment and still manifest when the rats are returned to their home cages. On the other hand, if a confinement time that lasts >30 min is conducted, the maximum effects of LiCl will occur and disappear within the compartment. Both explanations can be addressed in terms of contingency analyses (Cannon et al. 1975). Confinement periods shorter than the time required for the most severe effects of LiCl to appear increase the likelihood that such effects will be present in the absence of the conditioned stimulus (i.e., within the home cage), while longer periods increase the probability not only of lithium effects to occur inside the compartment but also of its disappearance in that same place. Inadequate contingency management during the presentation of stimuli is considered one of the main causes of interference in associative learning. Specifically, in taste learning experiments, pre-exposure to gastric malaise before its pairing with the taste stimulus has been observed to interfere with conditioned taste aversion (Domjan and Best 1977; Misanin et al. 1983). Similarly, when the conditioned stimulus remains accessible to animals even after the administration of the toxic agent, a taste preference is developed and interpreted as a "medicine effect" in which the conditioned stimulus is no longer associated with malaise but with recovery from illness (Green and Garcia 1971; Hasegawa 1981). Therefore, an adequate contingency-inducing CPA promotes optimal association between the context and gastric malaise.

However, CPA could be associated with the magnitude of peritoneal irritation and not with the toxicity or emesis induction per se. In this regard, it has been reported that many agents that induce abdominal pain, peritoneal irritation, and a writhing response fail to produce conditioned taste aversion (Grill 1985; Sakai and Yamamoto 1997), and subsequently fail to induce CPA. To ensure that the place avoidance was induced only by the i.p. injection of hypertonic LiCl (indicating high toxicity or emesis-eliciting information), we aimed to inject isotonic 0.15 M NaCl or hypertonic 0.4 M NaCl and then confine them in the black compartment to discard that the introduction of the needle or hypertonic solution administration could induce CPA. Moreover, it has been described that hypertonic NaCl results in irritation and causes an abdominal writhing effect (Lane et al. 1998). Neither the administration of isotonic nor hypertonic NaCl decreased exploration time in the black compartment during post-test, leading to the conclusion that CPA was established using LiCl and its gastric malaise effect.

As mentioned before, the administration of a muscimol/baclofen cocktail within the dorsal or ventral hippocampal portions impairs conditioned place avoidance, but such impairment differs depending on which subregion is inactivated. Thus, the ventral region appears to have a differential role in CPA formation induced by gastric malaise compared with the dorsal portion. Our results contribute to the description of the neuroanatomical substrates that underlie CPA. To our knowledge, the limited literature available does not take into account the hippocampal influence on this task but has already described the involvement and relevance of other brain structures, including the prelimbic cortex (Jiang et al. 2014), the periaqueductal gray matter, the basolateral complex of the amygdala (Zanoveli and Brandão 2008), or the anterior cingulate cortex (Gao et al. 2004). In general, these brain structures are typically associated with the processing of painful stimuli and adverse emotional reactions without considering contextual stimuli encoding, present and relevant for the CPA task. From this rationale, our findings contribute to understand this missed issue since we observed that the dorsal hippocampus, known for its relationship with spatial memory (Cimadevilla et al. 2005), is a region that naturally plays an important role in the establishment of CPA induced by exteroceptive stimuli, such as formalin administration and electric shocks. However, we provide experimental data suggesting that the dorsal hippocampus also contributes to CPA establishment when interoceptive aversive stimuli are used. Moreover, in addition to the structures studied by other works, we aggregate evidence that the ventral portion of the hippocampus, usually related to emotional aspects and specially activated in stressful or anxiogenic situations (Wang et al. 2019), is even more important for the development of a conditioned place avoidance induced by gastric malaise.

In general, the hippocampus is a brain structure highly involved in episodic memories. Remarkably, the dorsal region is related to spatial navigation (Pothuizen et al. 2004; Fanselow and Dong 2010) and the ventral portion contribute to anxiety-related behaviors (Kjelstrup et al. 2002; Jimenez et al. 2018). Our results suggest that the ventral hippocampus has a prominent role in CPA establishment induced by LiCl. In this regard, the ventral hippocampal region is related to emotion-related memory processing since pharmacological inhibition, electrolytic and excitotoxic lesions disrupt fear conditioning (Maren and Holt 2004; Rogers et al. 2006). Additionally, the ventral hippocampus has been widely related to anxiety and fear expression (Kjelstrup et al. 2002). Thus, we propose that inhibiting ventral hippocampal activity would probably lead to the abolition of anxiogenic states and the development of preference for the compartment previously associated with gastric malaise. This argument may be supported at least in part by the absence of such effect in the dorsal hippocampus, whose inactivation did not generate preference and only blocked CPA instead. These findings likely reflect, by one side, the amnesic properties of inactivating dorsal hippocampus and the failure to retrieve contextual or spatial information, and by the other side, the anxiolytic effects of interfering with the activity of the ventral hippocampus promoting exploration. Nevertheless, our experiments do not let us account for a possible dissociation of dorsal and ventral hippocampal participation in CPA.

These emotional responses may result from an amygdalar modulation since it has been reported that the basolateral amygdala projects directly to the CA1 subregion of the ventral hippocampus and modulates anxiety-related behaviors (Felix-Ortiz et al. 2013). Additionally, CPA induced by LiCl may be regulated by the amygdala since the administration of LiCl induces a glutamatergic response within the amygdala (Miranda et al. 2002; Guzmán-Ramos et al. 2012), modulating avoidance memory

consolidation in other brain structures such as the insular cortex (Guzmán-Ramos et al. 2010). However, it remains to be demonstrated that the amygdala modulates the ventral hippocampus's involvement in CPA induced by gastric malaise.

An important finding that emerged from our experiments is the participation of glutamatergic NMDA receptors in CPA development and maintenance. Our data are in contrast to other studies that conclude that hippocampal NMDA receptors have a crucial role in memory acquisition (Nakazawa et al. 2003; Yamada et al. 2017) since we found the administration of APV before conditioning did not affect CPA. However, intrahippocampal administration of APV into the dorsal and ventral regions after acquisition hinders CPA long-term consolidation induced by gastric malaise. This phenomenon agrees with extensive literature that reports the NMDA receptors importance for consolidating different types of memory, such as spatial and social and object recognition memory (Parsaei et al. 2016; Osorio-Gómez et al. 2019; Marcondes et al. 2020). A possible explanation for the differential effects observed between drug-administration times is the short half-life of APV, as it has been reported APV-induced plasticity inhibition lasts ~60 min (Ji et al. 2005). Nevertheless, administration of APV after CS–US impairs NMDA receptor activation, which constitutes the principal cellular mechanism for memory consolidation and maintenance (Cui et al. 2004). Calcium entry through NMDA receptors modulates several signaling pathways including, among others, CREB and elongation factors (Tran et al. 2007; Hoeffer and Klann 2009), promoting the synthesis of proteins required for setting up permanent synaptic changes (Steward and Schuman 2001; Igaz et al. 2002; Tran et al. 2007) that maintains memory.

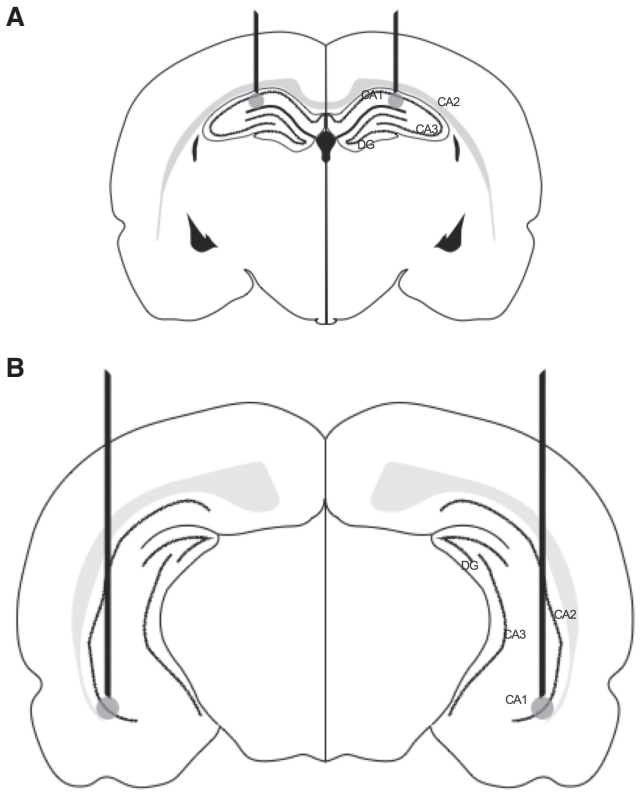

Figure 5. Histological representations of the microinfusion within the dorsal (*A*) and ventral (*B*) hippocampal regions.

Conclusions

The hippocampus is a critical brain structure involved in episodic memories. Importantly, structural and functional differences have been observed in the dorsal and ventral hippocampal regions. In this regard, we assessed the participation of both hippocampal regions in the formation of conditioned place avoidance induced by gastric malaise. Our results indicate that induction of a reliable CPA induced by LiCl administration is possible after one acquisition session and a period of 20 min of confinement. Our results also showed that CPA establishment involving contextual and visceral stimuli information depends on the dorsal and ventral hippocampal regions. Notwithstanding, the ventral region plays a predominant role in this type of CPA. In addition, activation of hippocampal NMDA receptors is required for long-term CPA memory maintenance.

Materials and Methods

All experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of the Instituto de Fisiología Celular (DOG159-20). Adult male Wistar rats weighing 260–280 g were used in this study, following the "International Guiding Principles for Biomedical Research Involving Animals." Animals were obtained from the Instituto de Fisiología Celular and were housed individually at 22°C in a 12-h light/12-h dark cycle starting at 7 a.m. Rats had ad libitum access to water and food.

Place conditioning paradigm

Apparatus design

Rats were trained in a custom-made acrylic three-chamber apparatus (95 × 25 × 50 cm). Each chamber is composed of different visual and tactile stimuli. Chambers were separated by two sliding guillotine doors. The middle compartment (15 × 25 × 50 cm) had gray walls and a solid gray floor. Conditioning compartments (40 × 25 × 50 cm) were black with stainless steel rods floor or white with a stainless-steel hole mesh floor. Apparatus was placed in a dim-light illuminated room with white noise. A video camera was mounted above the apparatus and all test sessions were recorded. The apparatus was thoroughly cleaned with 70% (v/v) ethanol after each session to avoid remaining olfactory cues.

General place conditioning procedure

For each session, rats were transported from the vivarium to the experimental room 1 h before the procedures. Animals were left in the same room for an additional hour at the end of each session. In the pretest session, the rat was placed at the middle compartment and the guillotine doors were opened to allow free exploration among chambers over 10 min. We used a biased design since animals prefer the black compartment; therefore, we trained rats to associate the gastric malaise-inducing agent with the preferred compartment. During conditioning session, animals were injected i.p. with 0.4 M LiCl (7.5 mL/kg) and confined to the black chamber for 10 min ($n = 6$), 20 min ($n = 8$), 40 min ($n = 7$), or 60 min ($n = 7$). Afterward, rats were returned to their home cage; 4 h later, rats were injected i.p, with 0.15 M NaCl (7.5 mL/kg) and confined to the white chamber for 10, 20, 40, or 60 min, respectively. The order of LiCl and NaCl administrations were counterbalanced, such that half of the animals were injected first with NaCl and confined to the white compartment and then received a LiCl injection and confined to the black compartment. On the post-test session, rats were placed at the middle compartment and the guillotine doors were opened to allow free exploration among chambers for 10 min. The behavioral analyses were performed by an experimenter blind to the treatment. We analyzed the time spent in each compartment during pretest and post-test sessions and calculated the percentage of time spent on each compartment. Changes in place avoidance were determined by a CPA score (CPA score = percentage of time spent during post-test in the preferred chamber – percentage of

time spent during pretest in the preferred chamber) (Rinaman et al. 2009).

Guide cannulae implantation

Using standard stereotaxic procedures, rats were implanted bilaterally with 9-mm (23G) steel cannulae aiming at the dorsal hippocampus (AP −3.6 mm, L ±2.5 mm, DV −2.0 mm from Bregma) (Fig. 5A; Paxinos and Watson 1998). For ventral hippocampus experiments, rats were bilaterally implanted with 11-mm (23G) steel cannulae aiming at the ventral hippocampus (AP −5.5 mm, L ±5.3 mm, DV −6 mm from Bregma) (Fig. 5B; Paxinos and Watson 1998). The cannulae were fixed to the skull using two screws with dental acrylic cement. Stylets were inserted to prevent clogging. Animals were allowed to recover from surgery for 6 d before behavioral protocols.

Microinfusion procedure

During the infusion session, stylets were removed, and injectors were inserted into cannulae aiming at the dorsal region extending 1 mm or at the ventral region extending 2 mm below the tip. Injection needles were connected via polyethylene tubing to 10 µL Hamilton syringes, driven by an automated microinfusion pump (Carnegie Medicine). A volume of 0.5 µL (0.25 µL/min) was injected per hemisphere in the ventral or dorsal hippocampus; the injectors were left for another minute to allow diffusion into the tissue. Infusion times were 15 min before the CS–US association or immediately after the CS–US association. Injected drugs were saline solution 0.9% (w/v) (SS; $n=7$, $n=8$; $n=8$, $n=8$), a $GABA_A$ and $GABA_B$ agonist cocktail (0.1 mM Muscimol and 1 mM Baclofen [McGlinchey and Aston-Jones 2018]; $n=6$, $n=6$; Sigma-Aldrich), or DL-2-amino-5-phosphonovaleric acid 10 µg/µL (APV; $n=8$, $n=8$; $n=6$, $n=7$; Tocris Bioscience). According to our results, a period of 20 min of confinement induces a robust CPA. Therefore, all pharmacological manipulations were conducted before or after 20-min association protocol.

Data and statistical analysis

The data obtained were tabulated, plotted, and analyzed with the GraphPad Prism 9 software. Data are reported as the mean ± SEM. One-way ANOVA was used to compare differences in place conditioning after different confinement times. Fisher's LSD test was used as a post hoc analysis. Two-way ANOVA was used to analyze differences in CPA scores after muscimol/baclofen administration into the ventral or dorsal hippocampus. Fisher's LSD test was used as a post hoc analysis. Two-way ANOVA was used to analyze differences in CPA scores in APV experiments. Additionally, we compared the mean CPA scores with a hypothetical value of 0 with a one-sample *t*-test to determine whether animals avoid or prefer the black compartment after conditioning or pharmacological manipulations. The accepted level of significance was P-value ≤ 0.05.

Animal use and procedures conformed to the National Institutes of Health (NIH) guide for the care and use of the laboratory animals (NIH publication no. 8023, revised 1978) and were reviewed and approved by the Animal Care Committee of the Instituto de Fisiología Celular.

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