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Involvement of the lateral habenula in fear memory

Laura Durieux¹ · Victor Mathis^{1,2} · Karine Herbeaux¹ · Marc–Antoine Muller¹ · Alexandra Barbelivien¹ · Chantal Mathis¹ · Rémy Schlichter³ · Sylvain Hugel³ · Monique Majchrzak¹ · Lucas Lecourtier¹

Abstract

Increasing evidence points to the engagement of the lateral habenula (LHb) in the selection of appropriate behavioral responses in aversive situations. However, very few data have been gathered with respect to its role in fear memory formation, especially in learning paradigms in which brain areas involved in cognitive processes like the hippocampus (HPC) and the medial prefrontal cortex (mPFC) are required. A paradigm of this sort is trace fear conditioning, in which an aversive event is preceded by a discrete stimulus, generally a tone, but without the close temporal contiguity allowing for their association based on amygdala-dependent information processing. In a first experiment, we analyzed cellular activations (c-Fos expression) induced by trace fear conditioning in subregions of the habenular complex, HPC, mPFC and amygdala using a factorial analysis to unravel functional networks through correlational analysis of data. This analysis suggested that distinct LHb subregions engaged in different aspects of conditioning, e.g. associative processes and onset of fear responses. In a second experiment, we performed chemogenetic LHb inactivation during the conditioning phase of the trace fear conditioning paradigm and subsequently assessed contextual and tone fear memories. Whereas LHb inactivation did not modify rat's behavior during conditioning, it induced contextual memory deficits and enhanced fear to the tone. These results demonstrate the involvement of the LHb in fear memory. They further suggest that the LHb is engaged in learning about threatening environments through the selection of relevant information predictive of a danger.

Keywords

Amygdala · Hippocampus · Prefrontal cortex · c-fos · Chemogenetic · Context

Introduction

The lateral habenula (LHb) plays a key role in the integration of basal ganglia and forebrain limbic information. It is implicated in functions such as reward prediction error, coding of negative motivational value, spatial memory, and subjective decision biases (Matsumoto and Hikosaka 2009; Stamatakis and Stuber 2012; Stopper and Floresco 2014; Mathis et al. 2015, 2018; Baker and Mizumori 2017). One of the main roles of the LHb is to process stressful situations (Stamatakis and Stuber 2012; Amo et al. 2014; Hennigan et al. 2015; Chou et al. 2016). LHb activation has been reported following stressful experiences such as electrical footshocks, immobilization, and tail pinch (Chastrette et al. 1991; Wirtshafter et al. 1994; Cullinan et al. 1995). Moreover, the LHb is a main afferent of the rostromedial tegmental nucleus (RMTg), a modulator of the activity of dopamine (DA) neurons, a structure also activated following repeated foot-shock delivery (Sanchez-Catalan et al. 2017; Li et al. 2019a, b). In addition, activation of afferent pathways to the LHb, from the globus pallidus, the ventral tegmental area (VTA) or the lateral hypothalamus (LH), promote avoidance behaviors (Lammel et al. 2012; Root et al. 2014; Lecca et al. 2017), whereas silencing of the LH–LHb pathway impairs escape behavior (Lecca et al. 2017). Altogether, these data indicate that the LHb plays a prominent role in the behavioral adaptation to aversive situations. LHb neurons were also reported to progressively increase their activity during the presentation of a conditioned stimulus (CS) that precedes footshock delivery (unconditioned stimulus, US) during avoidance learning (Truscel et al. 2019) and Pavlovian fear conditioning (Wang et al. 2017), while maintaining US responding, suggesting it is involved in the encoding of the predictive relationship between the CS and the US. However, only few studies (Wang et al. 2013; Song et al. 2017; Barrett and Gonzalez- Lima 2018) shed light on its contribution to Pavlovian fear memory. Such a contribution can also be expected

given that in rodents the LHb is directly or indirectly connected with the main structures involved in fear conditioning. i.e., the medial prefrontal cortex (mPFC), the amygdala, and the hippocampus (HPC). If contextual fear learning is sensitive to hippocampal manipulation, fear learning to a discrete CS only requires the HPC during trace fear conditioning paradigms, when the CS and the US are separated by an empty temporal interval named the trace interval (Misane et al. 2005; Chowdhury et al. 2005; Esclassan et al. 2009). Importantly, the mPFC is also required for trace conditioning (Gilmartin and Helmstetter 2010; Guimaraes et al. 2011; Gilmartin et al. 2013) as well as for contextual fear learning (Gilmartin and Helmstetter 2010). Anatomically, the LHb receives direct projections from several subregions of the mPFC (Kim and Lee 2012); recently, a connection with the amygdala has been described in mice (Kim and Han 2016; Zhou et al. 2019), although other anatomical investigations fail to report it (e.g. Zahm and Root 2017), so this needs to be confirmed in the Rat. Although the LHb and dorsal HPC (dHPC) are not directly connected, electrophysiological recordings in the head-restrained as well as in behaving rats, demonstrated coherent activity between these two structures (Aizawa et al. 2012; Goutagny et al. 2013), strongly suggesting they exchange information. These findings, along with the known role of the LHb in HPC-dependent spatial memory (Goutagny et al. 2013; Mathis et al. 2015, 2018) and in mPFC-dependent working memory (Mathis et al. 2016), suggest that the LHb could contribute to both CS trace and contextual fear learning. To test this hypothesis, we used a trace conditioning protocol with a trace interval (30 s) long enough to allow fear conditioning to both the context and the tone (Detert et al. 2008). In a first experiment, we questioned whether the LHb was part of the network sustaining trace fear conditioning acquisition by quantifying the expression of the c-Fos protein in the habenular complex, the HPC, the mPFC, and the amygdala; we analyzed the presence of co-activation among these structures using a factorial analysis design. In a second experiment we used a chemogenetic approach, with a modified muscarinic hM4(Gi) receptor, to perform LHb inhibition during conditioning and assessed its effects on fear elicited by re-exposure to the context and to the CS. In addition, we studied in the same rats the effects of LHb chemogenetic inactivation on plus-maze behaviors and home cage locomotor activity, both previously shown to be sensitive to LHb inactivation (Mathis et al. 2015).

Materials and methods

Animals

This study, authorized by the French authorities (APAFIS#7114), required 66 male Long-Evans rats (250–350 g; Janvier Labs, France). They were housed in pairs on a 12 h light/dark cycle (lights on at 7:00 A.M.) with ad libitum access to food and water, controlled temperature (~ 23 °C), and a hygrometry of about 55%. Animals were distributed as follows: experiment 1: c-Fos study (n = 18); experiment 2: electrophysiological validation of the DREADD technique (n = 4) and behavioral study (n = 44). Testing took place between 9:30 am. and 2 pm.

Fear conditioning

Four conditioning chambers (25 cm x 27 cm x 18 cm) located in a lit- (6 lx) and sound-attenuating box (57 cm x 38 cm x 38 cm, Campden Instruments) were used. Chambers were made of transparent plastic with a loudspeaker fitted on one of the sidewalls, a transparent ceiling, and a grid floor (parallel 0.3 cm diameter stainless-steel bars spaced 0.8 cm apart) above a sawdust tray. A camera (MCT- 210 MS, OptoVision, Toulouse, France) was fitted inside each box, above the center of the chamber, and monitored the entire chamber from the top through a 2.45 mm-wide angle lens. These chambers were used as conditioning context whereas triangular plexiglass boxes with one gray wall, one black and white striped wall, and a smooth white floor could be placed within the chambers and served as a new context. Chambers were cleaned with water between successive rats. Tones and electric shocks were delivered through a computerized interface (Med Associates Inc., St Albans, VT, USA). Fear conditioning (38 min-long session) was conducted as follows: rats were placed in the conditioning chamber and, after a 3 min baseline with no event, received six CS and US presentations (CS: 15 s tone, 4000 Hz, 10 dB above background; US: 0.5 mA before scrambling, 0.8 s, through the

grid floor) with the US delivered 30 s after CS offset; the 6 presentations were made with a 4 min 20 ± 26 s intertrial interval. The automatic measurements of freezing behavior, defined as the suppression of all visible movements except those needed for breathing –and used as a measure of fear–, were performed as previously described (Marchand et al. 2003). A set of procedures written under Excel® Visual Basic®, allowed the computation of the percentage of time spent freezing over blocks of selected duration.

Experiment 1.

C-Fos expression related to fear conditioning

A schematic representation of the experimental procedure is given in Fig. 1a. Tissue preparation and section processing Rats were conditioned as described above (Fear conditioning, FC, condition), or remained in their home cage (HC) to assess baseline c-Fos expression. An additional group of rats was exposed to the conditioning chambers with tones delivered exactly as during conditioning but without applying electric shocks (No shock, NS). Ninety minutes after the beginning of the session, rats were deeply anesthetized with pentobarbital overdose (120 mg/kg, i.p). Following intracardiac perfusion of phosphate-buffered saline (PBS, 0.1 M) and then 4% paraformaldehyde (PFA)-PBS solution (pH 7.4; 4 °C), brains were removed, post-fixed in 4% PFAPBS (4 °C, 48 h), transferred into a 0.1 M PBS–20% sucrose solution (4 °C, 48 h) and subsequently frozen (isopentane, – 40 °C, 1 min). Serial 40 μ m-thick free-floating sections were cut in the coronal plane at – 20 °C and collected then stored in cryoprotectant at – 20 °C. Immunohistochemistry Sections were first rinsed three times during 10 min in PBS before being soaked for 1 h in 5% normal horse serum in PBS/0.5% Triton X– 100. They were subsequently transferred into the primary anti-Fos rabbit polyclonal antibody (1:750, polyclonal rabbit antibodies; SYSY; ref: 226 003, Synaptic System) solution for 20 h at room temperature, and then in a buffer solution containing biotinylated goat antirabbit secondary antibody for 1 h (1:500, Biotin-SP-conjugated affiniPure Goat anti-rabbit IgG, ref: BA1000, Vector). Staining was revealed with the avidin–biotin peroxidase method (Vectastain ABC kit, PK 6100; Vector Laboratories, Burlingame, CA, USA). After two PBS and one Tris (0,6%, pH 7,6) 10 min washes, sections were exposed for 8 min to a revelation solution (Kit DAB, SK 4100, Vector Laboratories) containing DAB (3,3'-diaminobenzidine) and H₂O₂ (hydrogen peroxide). Sections were then dropped on gelatinated slides, dried for 24 h, dehydrated by incrementally concentrated alcohol baths (70%, 90%, 95%, 100%, 100%), covered with Clearify (Americain MasterTech Scientific), fixed on microscopic slides with Diamount (Diapath S.P.A), and dried for 48 h, protected from light. Quantification Quantification of c-Fos + cells were performed (see Supplementary information and Supplementary Figs 1–6) bilaterally in mPFC [prelimbic (PRL), infralimbic (IL), and anterior cingulate (ACC)], basolateral (BL) and lateral (LA) nuclei of the basolateral complex and central nucleus (CeA) of the amygdala, habenular complex, dorsal and ventral HPC (dHPC and vHPC respectively). The habenular complex was divided into a medial (MHb) subregion, and a lateral (LHb) subregion, the latter being further divided into a lateral (LHbL) and a medial (LHbM) part; in addition, we considered separately the LHb subregion in its most rostral part (rLHb) where LHbL and LHbM do not appear yet according to Paxinos and Watson (Paxinos and Watson 2007). Both the dHPC and vHPC were divided into ammonic fields 1 (CA1) and 3 (CA3), and dentate gyrus (DG). Countings were performed using a semi-automated method with ImageJ (Free License, Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA; for more details see Supplementary information and Supplementary Fig. 7) and expressed as mean number of c-Fos positive (c-Fos +) cells by mm². The values of both hemispheres did not differ so that they were pooled (Supplementary Fig. 8). Factorial analysis Factorial analysis was conducted using the Statistica software. It was performed to unravel homogeneous functional networks, i.e. regional activities that are consistently correlated in FC and NS groups. The aim of this kind of analyses is to compress a large number of correlations into fewer factors. These factors are created through the computation of the correlation matrix of initial variables, without any prior (hypothesis-driven) input from the experimenter and represent ad hoc variables that show strong correlations with specific clusters of heavily intercorrelated observed variables. In addition to this ability to summarize an overwhelming

number of covariations into fewer factors, this analysis is considered a way to unfold the underlying structure organizing these correlations. Applied to functional imaging studies, factorial analysis helps to unravel homogeneous functional networks (i.e. regional activities that are consistently correlated), as previously shown (Veening et al. 2009; Ali et al. 2017). Although this analysis initially extracts a high number of factors, only a set amount is classically described, as factors show diminishing relevance: they are determined in a series, each new one trying to bind residual correlations that had not yet been explained by preceding factors. The number of factors retained is determined by the observation of each factor's eigenvalue, describing the proportion of total variance that is explained by the adjunction of a supplementary factor relative to initial variables. For instance, an eigenvalue equal to 1 indicates that the corresponding factor explains exactly as much dispersion as one of these variables. To this aim, one of the most widely used criteria is the Guttman–Kaiser's: only factors with eigenvalues superior to 1 are conserved, to keep modeling of raw data from redundancy. When described, factors are associated with factor loadings of each observed variables, which correspond to correlations of the latter with the former, allowing for the description of the computed cluster, these correlations being considered strong above 0.6 or under -0.6 . Computation of factorial analysis was conducted using varimax rotation, which increases discrepancies between these factor loadings on a given factor, thus clarifying the variables critically contributing to this factor's extraction.

Experiment 2.

Effects of chemogenetic LHb inactivation on fear memory, anxiety, and locomotor activity A schematic representation of the experimental procedure is given in Fig. 1b. Surgery Rats underwent surgery under isoflurane anesthesia [4% for induction in an induction box (3 min), 1.5% throughout surgery] delivered in O₂. Prior to surgery, they received a painkiller (meloxicam, 1 mg/kg, s.c.). After their head was shaved, rats were secured into a stereotactic apparatus, covered with an aluminum blanket to prevent hypothermia, and lidocaine (0.02 mg in 0.1 ml, s.c.) was injected at the incision location. Following incision, burr holes were drilled above the LHb. AAV8–CamKII–hM4(Gi)–mCherry [Viral Production Unit, Universitat Autònoma de Barcelona (VPU); (Piedra et al. 2015)] or phosphate-buffered saline (PBS) as a Sham operation was bilaterally injected into the LHb, by means of a 33–gauge Hamilton syringe, at the two following locations and volumes: (1) anteroposterior (AP) = -3.3 mm from Bregma, mediolateral (ML) = ± 0.7 mm from the midline of the sagittal sinus, dorsoventral (DV) = -4.5 mm from dura (0.2 μ L); (2) AP = -3.5 mm from Bregma, ML = ± 0.7 mm from the midline of the sagittal sinus, DV = -4.4 mm from dura (0.15 μ L). Among the 44 rats of the behavioral study, 22 were injected with the viral vector and 22 with PBS. Once the injections terminated the syringe was left in place for 7 min and then slowly removed from the brain, before the skin was stitched. Animals were then placed in cages under a heating lamp until complete awakening. Rats received a second injection of painkiller (meloxicam, 1 mg/kg, s.c.) the following day. After a one week recovery period in individual cages, the same pre-surgery pairs of rats were housed together again. Experiments started three weeks after surgery (Fig. 1b). In vitro electrophysiological recordings The efficacy of the chemogenetic strategy was first tested using patch-clamp recordings. Three rats underwent the surgical procedure described above, to express hM4(Gi) receptors within the LHb, and one rat was used to assess the consequences of Clozapine N–Oxyde (CNO) administration on the excitability of LHb neurons not expressing the hM4(Gi) receptor. Acute slice preparation Rats were deeply anesthetized with an i.p. injection of a mixture of ketamine (82.5 mg/kg, Imalgène 1000, Merial) and xylazine (11 mg/kg, Rompun 2%, Bayer). Intracardiac perfusion was performed with 300 ml of cold (0–4 °C) sucrose–based artificial cerebrospinal fluid containing: 248 mM sucrose, 11 mM glucose, 26 mM NaHCO₃, 2 mM KCl, 1.25 mM KH₂PO₄, 2 mM CaCl₂, 1.3 mM MgSO₄, and 2.5 mM kynurenic acid (bubbled with 95% O₂ and 5% CO₂). After 15 min, rats were decapitated, their brain removed and placed in a matrix allowing to split it in two at the level of the longitudinal sulcus and to remove the posterior part using a razor blade. Each brain half was mounted on the vibratome stage (VT1200S; Leica, Nussloch, Germany) by gluing the posterior end, the median plan facing the blade. This allowed us to perform transverse slices (thickness: 300 μ m) and to start slicing from the

midplane of each brain half. Slices were stored at room temperature in a chamber filled with artificial cerebrospinal fluid (aCSF) containing: 126 mM NaCl, 26 mM NaHCO₃, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, 2 mM MgCl₂, and 10 mM glucose (bubbled with 95% O₂ and 5% CO₂; pH = 7.3; 310 mOsm measured). Patch-clamp recordings Slices were transferred to a recording chamber and continuously superfused with oxygenated aCSF at 34 °C. Lhb neurons, selected based on the presence of mCherry fluorescence (hM4 neurons) or on its absence (no hM4 neurons), were recorded in the whole-cell configuration of the patch-clamp technique. Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, Edenbridge, UK) with a P-1000 puller (Sutter Instruments, Novato, CA, USA). They were filled with a solution containing 140 mM KCl, 2 mM MgCl₂, 10 mM HEPES, 2 mM MgATP, (pH = 7.3, adjusted with KOH; osmolarity, 310 mOsm, adjusted with sucrose) and had final tip resistances of 3.5–4.5 MΩ. Current-clamp experiments were performed with a Multiclamp 700 A amplifier (Molecular Devices, Union City, CA, USA) and recorded with Win-WCP or WinEDR softwares (John Dempster, University of Strathclyde). Recordings were performed at a holding current allowing to maintain the resting potential at ca. –60 mV. In the first set of experiments we investigated, on Lhb neurons including hM4(Gi) receptors (n = 2 rats), the consequences of bath perfusion of CNO (5 μM) on firing frequency in response to intracellular injection of 1 s-lasting current pulses of increasing amplitude (from 0 to 240 pA, increments of 20 pA). Responses were recorded twice, once before and once following CNO bath application. In the second set of experiments, we investigated the consequences of local application of 1 μl CNO (500 μM) on spontaneous firing frequency of Lhb neurons, with (n = 1 rat), and without (n = 1 rat) hM4(Gi) receptor expression, by means of puff application through a second pipette positioned just above the recorded Lhb neurons. Spike frequency was analyzed with Clampfit 10.2 (Molecular Devices, Union City, CA, USA). This was done to be more precise and selective of the recorded neurons and to increase the chance to observe a recovery of neuronal firing to baseline levels, through the local administration of a very small amount of CNO solution. Behavioral study Drug treatments CNO (1 mg/kg; freshly prepared before each experiment and dissolved in 0.9% NaCl–0.5% DMSO), or vehicle (Veh, 0.9% NaCl–0.5% DMSO) were administered intraperitoneally. Sixteen hM4 animals and 16 Sham animals were administered CNO (hM4–CNO group and Sham–CNO respectively). Six rats of each surgery condition (hM4–Veh group and Sham–Veh group) were administered vehicle, hM4–Veh animals serving to control for possible adverse effects of the administration of the viral construct and subsequent inclusion of hM4 receptors at the cellular membrane. The Sham–CNO group was used to control for potential adverse effects of CNO (Ilg et al. 2018; Manvich et al. 2018; Campbell and Marchant 2018) in our conditions. Fear conditioning The protocol took place over four consecutive days. Rats were subjected to each session in the same chamber. On day 1 (Conditioning), rats were conditioned as described above. On day 2 (Context test; 15 min–long), they were placed in the conditioning chamber to assess contextual fear. On day 3 (New context exposure; 15 min–long), rats were placed in the new context, the CS test context, to reduce baseline fear before CS fear assessment (Jacobs et al. 2010). On day 4 (CS test; 26 min–long), after a 2 min baseline in this context with no event, five CS were delivered with a 4 min interval. Rats received CNO or Veh on day 1, 30 min before the start of conditioning, and were administered Veh on each subsequent testing day, 30 min before the start of the session. Freezing scores were averaged on periods of interest on day 1 (the 3 min before the first tone presentation serving to assess baseline freezing), day 2 and day 3 (the 5 first min to capture the primary reaction to the conditioning context, and to the new context, respectively), and day 4 (the 15 s preceding the first tone being used to assess baseline, and the five 15 s tone presentations being used to assess fear conditioned to the CS). Elevated plus-maze It was made of black Plexiglas, elevated 73 cm above the floor, and consisted of four arms (50 cm × 10 cm), two comprising 40 cm–high walls (closed arms) and two comprising 1.5 cm–high borders (open arms). Light intensity was 10 lx in open arms, 7 lx at the center of the maze, and 2.5 lx in closed arms. The maze was cleaned with water and 70% ethanol between each rat. Thirty min after CNO or vehicle injections, rat were put in the maze for 5 min. The data analyzed were the total number of visits in the four arms, the number of visits in the open arms (in the percentage of the total number of visits), and the time spent in the open arms (in the percentage of the total time spent in the four

arms). Locomotor activity Locomotor activity was assessed in the home cage (HC) by means of two infrared light–beams perpendicular to the width of the cage, placed 4.5 cm above floor level and 28 cm apart along the length of the cage. The consecutive interruptions of both light beams were counted as longitudinal crossings, whose numbers were monitored and saved in 15 min bins. Following a 1 h baseline, rats were administered CNO or Veh, and activity was recorded for an additional 2 h. Histology Tissue preparation and section processing were conducted as described in experiment 1, and sections were mounted on gelatin-coated slides with a DAPI–fluoromount medium. Observation of the spread of hM4(Gi) receptor expression (visualization of mCherry fluorescence) was performed with a Zeiss Apotome microscope (Zeiss, Muenchen, Germany). Statistical analyses For Experiment 1, freezing scores during each 15 s tone presentation and c–Fos + cells densities in the habenular complex were analyzed using one-way ANOVAs with repeated factor (Trial and Subregion, respectively). For the analyses of electrophysiological data from Experiment 2, one– and two–way ANOVAs (with Group as the factor and Current pulse amplitude or Time as the repeated measures) was used. For the analyses of all behavioral data from experiment 2, Group was used as the factor. For fear conditioning, freezing scores during baseline (day 1), reexposure to the conditioning context (day 2), and exposure to the new context (day 3) were analyzed using one–way ANOVAs. Twoway ANOVAs were used to compare groups through conditioning (factor Trial, freezing scores during each 15 s tone presentation, day 1) and during the test of conditioned fear to the CS (factor Tone, 15 s before the first CS presentation vs during CS, day 4). Data collected in the plus–maze and in the home cage were analyzed using one– and two–ways ANOVAs (with Time as the repeated measure), respectively. Post hoc tests used the Newman Keuls multiple range test (except when specified) when appropriate. Values of $p < 0.05$ were considered significant.

Results

Experiment 1

The increase in freezing scores to the tone during the session (Trial, $F_{5,15} = 18.85$, $p < 0.0001$, first tone vs second tone, $p < 0.05$, second tone vs third tone, $p < 0.01$, and asymptotic score— $90\% \pm 6.3$ —thereafter) indicated that FC rats were successfully conditioned. Although a significant Trial effect was found in NS condition ($F_{7,35} = 4.09$, $p < 0.01$), the increase in behaviors coded as freezing by our automated system only reached its low ($26 \pm 9.4\%$) asymptotic level during the fifth tone presentation ($p < 0.05$ vs the previous ones), very unlikely to be indicative of the onset of unconditioned fear responses to this stimulus. As compared to the home cage, NS and FC conditions both induced low to marked c–Fos expression in all the investigated structures (Supplemental Table 1). In the habenular complex (Fig. 2 a, b), c–Fos + cells densities differed according to the subregion of both NS and FC rats (HC, $F_{3,15} = 1.56$, $p > 0.2$; NS, $F_{3,21} = 31.17$, $p < 0.0001$; FC, $F_{3,9} = 17.38$, $p < 0.001$). Post hoc comparisons indicated that the density of c–Fos + cells were higher in rLHb and LHbM than in LHbL and MHb in NS ($p < 0.01$ at least), and FC ($p < 0.01$ at least) conditions, and higher in the rLHb than in LHbM only in NS condition ($p < 0.01$). Factorial analyses of c–Fos data are shown in Table 1. In the NS condition, analysis of eigenvalues led to the conservation of a 3 factor–model accounting for 87.85% of the total variance observed. Factor 1 explained 65.34% of this variance and included positive loadings of the whole mPFC, the rLHb and LHbM, the whole CA3 region (ventral and dorsal), and the vDG. Factor 2 explained 15.8% of the total variance and included positive loadings of the CeA and LA, MHb, dDG, and vCA3. Factor 3 explained 6.73% of the total variance and included positive loadings of BL, LHbL, and vCA1. In the FC condition, analysis of eigenvalues led to the conservation of a 3 factor–model accounting for nearly 100% of the variance observed. Factor 1 explained 51% of the total variance and included positive loadings of the entire mPFC, the MHb, and the entire dHPC, and negative loadings of the BL and rLHb. Factor 2 explained 29.66% of the total variance and included positive loadings of CeA, LA, and LHbL, and negative loadings of LHbM and MHb. Factor 3 explained 19.34% of the total variance and included positive loadings of dCA3 and vCA3, and negative loadings of vCA1 and vDG. As illustrated in Fig. 2c, NS, and FC seem to engage the structures of interest but within distinct networks.

Experiment 2

CNO reduced the excitability of hM4(Gi)-containing LHB neurons CNO reduced action potentials (AP) firing in response to pulses up to 100 pA ($F_{12,48} = 4.25$, $p < 0.001$; Fig. 3a). For example, the AP frequency in response to 100 pA current pulses in the presence of CNO represented $46 \pm 14\%$ of the frequency recorded in the control condition. Note that under strong current pulses injection (> 100 pA) the inhibitory effect of CNO was overcome (see traces with 240 pA injection in Fig. 3b). To control for possible nonspecific effects of CNO, we examined the consequences of local puff applications of high CNO concentration (500 μ M) on the spontaneous firing of LHB neurons with (hM4; $n = 1$ rat; $n = 4$ neurons) and without (no hM4; $n = 1$ rat; $n = 5$ neurons) hM4(Gi) receptors. Whereas CNO significantly reduced the firing frequency of hM4 neurons, it had no effects on the firing of no hM4 neurons (Time X Group, $F_{7,49} = 3.83$, $p < 0.01$; $p < 0.05$ at least, vs no hM4, Duncan post-hoc test; Fig. 3 c, d). Altogether, these results indicate that the effect of CNO was specific to neurons expressing hM4Gi receptors and strongly suggest that such an effect accounts for the behavioral alterations observed in hM4-CNO animals. Histology and groups size for the behavioral study Only rats in which mCherry expression was bilaterally mainly present in the LHB were kept. In these rats, hM4(Gi) receptors were expressed within the LHB in its entire rostrocaudal extent in both its lateral and medial parts (Fig. 4). In few of those rats, the expression of hM4 receptors also slightly impinges on the underlying paraventricular thalamic nucleus (PVT), particularly in its posterior part. Five hM4 rats were discarded because hM4(Gi) receptor expression spread to other surrounding structures. In addition, the two groups of rats which were administered Veh (hM4-Veh and Sham-Veh) were pooled into a single Ctl-Veh group after verification that their performances did not differ (Supplementary Information). Therefore, groups were composed as follows: hM4-CNO ($n = 11$), Sham-CNO ($n = 16$), Ctl-Veh ($n = 12$) and indicated as such in the figures. LHB inactivation altered conditioned fear memory Conditioning. All groups showed freezing scores close to 0% during baseline ($F_{2,36} = 0.90$, $p > 0.4$), and a similar increase of freezing to the tone during the session (Fig. 5a). Indeed, the analysis indicated no significant effect of Group ($F_{2,36} = 1.44$, $p > 0.2$), a significant effect of Trial ($F_{5,180} = 20.32$, $p < 0.0001$), and no significant Group x Trial interaction ($F_{10,180} = 1.32$, $p > 0.2$). This suggests that LHB inactivation did not affect the rate of fear learning. Context test (Fig. 5b). Reexposure to the training context-induced freezing behavior, but hM4-CNO group showed lower freezing score than the two other groups, the latter displaying similar freezing scores (Group, $F_{2,36} = 3.86$, $p < 0.05$; hM4-CNO vs Ctl-Veh and Sham-CNO, $p < 0.05$ for each comparison). This suggests that LHB inactivation during conditioning impaired conditioned fear of the training context. New context exposure (Fig. 5c). Freezing scores were low in all groups and of a similar level (Group, $F_{2,36} = 0.65$, $p > 0.5$), suggesting that exposure to this new context did not induce generalized contextual fear, whatever the group. CS test (Fig. 5d). All groups showed similarly low freezing scores during baseline and enhanced freezing during CS presentation (Tone, $F_{1,36} = 54.64$, $p < 0.0001$), especially in the hM4-CNO group (Group, $F_{2,36} = 5.88$, $p < 0.01$; Tone x Group, $F_{2,36} = 5.18$, $p < 0.05$). Post hoc comparisons indicated that hM4-CNO rats showed significantly more freezing than the two control groups only during CS presentation ($p < 0.05$). This suggests that, if all groups displayed fear to the CS, LHB inactivation during conditioning enhanced such fear response. LHB inactivation induced mild anxiety and increased locomotor activity In the elevated plus-maze, LHB inactivation did not affect the total number of arm visits (Group, $F_{2,36} = 1.29$, $p > 0.2$; Fig. 6a), nor did it impact the percent time spent in the open arms, despite a tendency (Group, $F_{2,36} = 2.13$, $p = 0.13$; Fig. 6b right). However, the percent of open arms entries were lower in the LHB inactivated group than in the two other groups (Group, $F_{2,36} = 4.21$, $p < 0.05$ for each comparison; Fig. 6b left). With regard to home cage locomotor activity, there was no difference during baseline (Group, $F_{2,36} = 0.89$, $p > 0.4$; Time, $F_{3,108} = 12.09$, $p < 0.0001$; Group x Time interaction, $F_{6,108} = 0.22$, $p > 0.9$; Fig. 6c). LHB inactivation induced marked hyperactivity (bins 5-12; Group: $F_{2,36} = 38.70$, $p < 0.0001$; Time: $F_{7,252} = 16.28$, $p < 0.0001$; Group x Time interaction: $F_{14,252} = 11.36$, $p < 0.0001$). Noteworthy, this hyperactivity peaked 30 min following CNO administration, further validating our strategy to perform behavioral testing 30 min following CNO administration, and lasted approximately 75 min (Fig. 6c). Together, these results indicate that chemogenetic LHB inactivation

induced only mild anxiety and a marked behavioral activation in a familiar (i.e. home cage), but not an unfamiliar –and anxiogenic– (i.e. the elevated plus maze) environment. These behavioral alterations have already been reported following LHB inactivation (Mathis et al. 2015).

Discussion

This study aimed to assess whether the LHB contributes to fear memory formation using a long trace fear conditioning procedure. Our results indicate that it participates to both contextual and CS memories formation. Notably, whereas LHB inactivation did not affect fear expression during learning, demonstrating that it did not impair the ability of the rats to perceive the footshocks, it resulted in poor freezing levels upon re-exposure to the context, consistent with contextual memory deficit, whereas it exacerbated fear response to the CS, which is evocative of CS memory enhancement. It is unlikely that those effects resulted from partial inactivation of the adjoining paraventricular thalamic nucleus (PVT); in rats, PVT inactivation before conditioning did not impact fear retention (Padilla-Coreano et al. 2012); also, PVT inactivation during a conditioning phase similar to ours induced in mice a decreased freezing during the CS retention test (Penzo et al. 2015), whereas here hM4–CNO rats showed increased freezing during the CS test. The contextual memory deficits induced by LHB chemogenetic inhibition can be discussed according to the two main processes leading to contextual fear conditioning: context encoding per se, and the association between the context and the aversive event. Context encoding rapidly takes place during conditioning through the elaboration of a conjunctive representation of the various cues of the conditioning chamber, a process mainly dependent on the dHPC (Rudy et al. 2002). An interaction between the LHB and dHPC might be critical to the proper encoding of contextual information. Such a view is strengthened by the presence of highly correlated metabolic activations in these structures during contextual fear conditioning (González-Pardo et al. 2012), as well as the depiction of electrophysiological communication between them (Goutagny et al. 2013; Aizawa et al. 2013), including in a task involving spatial information processing (Goutagny et al. 2013). Strikingly, in parallel with contextual memory deficits, hM4–CNO rats were able to perform and memorize the CS–US association. LHB neurons were recently shown to increase CS activity while continuing to be activated by the US during aversive classical conditioning (Wang et al. 2017) and avoidance learning (Trusel et al. 2019) suggesting that the LHB may be involved in the formation of CS-US association supporting predictive learning, at least when the CS is a perfect predictor of the US according to both its temporal and contingent relation with the US. In those conditions and based on this assumption, an alteration of LHB function may be expected to alter learning about the predictive value of the CS. However, LHB lesions were shown to facilitate avoidance learning (Song et al. 2017) and contrasting effects of its lesion were reported on CS fear memory (unaffected, Heldt and Ressler 2006; Song et al. 2017; altered fear memory to both the CS and the context, Wang et al. 2013). The recent demonstration of coexistence of two distinct populations of LHB neurons displaying opposite responses to footshocks indicates that LHB encoding of aversive cues involves a complex excitation/inhibition process (Congiu et al. 2019), and our current results showing enhanced fear conditioned to a trace CS in hM4–CNO rats suggest a more complex role of the LHB in predictive learning. Conditioning protocols where the Pavlovian law of temporal contiguity is manipulated –from the introduction of a long time interval to a complete lack of contingency between the CS and the US– typically lead to an increased fear to the context at the expense of the CS. This phenomenon indicates the onset of overshadowing of the CS by the context, a process commonly thought to rely on competition between potential predictors at the time of conditioning (Rescorla 1968; Rescorla and Wagner 1972). The fact that control animals (Ctl–Veh and Sham–CNO) displayed higher levels of freezing to the context than to the CS confirms the overshadowing effect in our long trace protocol. It is likely that an impaired ability to encode the context has led to a lack of competition with the CS, resulting in the ability of the latter to elicit increased fear responses in hM4–CNO rats. The Rescorla–Wagner model (Rescorla and Wagner 1972) posits that differences in the emotional value acquired by the different cues is based on differences in the perceived salience of the US and of those cues. The lack of alteration of the rate of fear learning in hM4–CNO rats suggests unaltered stimuli salience

processing; therefore, the role of the LHb may be to compute the relative pertinence of potential predictors (to shed light on the strongest one) and favor –or inhibit– their association with the aversive event. The reduced contextual- and enhanced CS-conditioned fear observed in hM4-CNO rats might thus be the consequence of an alteration of this computation. Interestingly, opposite changes in the acquired emotional value of contextual and CS cues were previously reported by Calandreau and collaborators (2005). Indeed, those authors have shown that LA inactivation during conditioning using a delay procedure (i.e. no trace interval between the CS and the US, leading to a strong overshadowing of contextual cues by the CS), reduced fear to the more predictive CS and enhanced fear to the less predictive context. Our results suggest that the LHb might modulate, along with the LA, fear learning according to the predictive value of competing cues. The c-Fos experiment results, rather than opposing two potential roles of the LHb in context encoding vs. association between stimuli, seem to be congruent with both, although with some anatomical peculiarities. First, the factorial analysis conducted in the NS and FC conditions led to different distributions of the investigated structure among factors. This indicates that even though neuronal activations were observed in the same structures in both conditions, as expected by the fact that all rats were exposed to novelty (see Milanovic et al. 1998; Radulovic et al. 1998; Cho et al. 2017), the onset of functional associations in these networks can be unraveled through correlational analysis of data. Specifically, the analysis of the FC group led to the extraction of 3 factors gathering neuronal ensembles classically described as sustaining different facets of information processing during fear conditioning. The first factor encompassed the whole dHPC and mPFC subregions, as well as the BL; it may represent the network implicated in contextual information processing (see for review Maren et al. 2013). However, the BL shows a negative loading on this factor, even though it has been repeatedly reported to be the main locus of association between the context representation and the US in contextual fear conditioning (see for review Duvarci and Pare 2014). Thus, this factor likely underlines cognitive facets of information processing in contrast with associative and/or emotional ones. Such an interpretation is congruent with the involvement of both the mPFC and the dorsal HPC in broad cognitive functions (see for review Fanselow and Dong 2010; Connor and Gould 2016). The second factor, characterized by heavy loadings of the LA and the CeA, may sustain the associative process as well as the onset of fear responses (see for review Duvarci and Pare 2014). Indeed, if CS–US association mainly takes place in the LA, the latter also supports context–US association (Calandreau et al. 2005). The mild positive loading of the BL on this factor might reflect associations between the US and its predictors. The third factor, characterized by heavy loadings on the whole ventral HPC, likely reflects emotional processes (Fanselow and Dong 2010). However, this factor puts forward CA3 activations in both dorsal and ventral portions of the HPC, in opposition to ventral DG and CA1 activations. This subfield of the HPC has historically been proposed as a relevant substratum to the encoding of polymodal representations such as a context (Marr 1971). Thus, this factor might also underline the processing of contextual information. The fact that the BL (along with the CeA) also displays a mild positive loading on this factor suggests that it might represent contextual computations relevant to fear conditioning, in contrast with factor 1. Of utmost interest is the presence of habenular subregions within these ensembles. The association of the MHb with factors 1 and 2 suggests it contributes to both cognitive facets of information processing, i.e. associative learning and the onset of fear responses. The implication of the MHb in trace fear conditioning has not yet been investigated. This region in rodents (Yamaguchi et al. 2013; Zhang et al. 2016; Geng et al. 2019), or its equivalent in zebrafish (the dorsal habenula; Agetsuma et al. 2010), modulate fear responses, although Hsu et al. (2016) found no contextual fear conditioning deficits following its lesion. The LHb is also included in these two factors, but with regional specificity. If the inclusion of its rostral part in factor 1 suggests its contribution to the cognitive aspects of fear conditioning, the inclusion in factor 2 of the LHbM and the LHbL, along with the LA and CeA, suggests they contribute to the associative process and/or to the onset of fear responses. The memory deficits observed following LHb inactivation appear in accordance with the position of the different LHb subdivisions within the clusters of structures revealed by the factorial analysis. Contextual memory deficits are in accordance with rLHb inactivation and the consecutive disturbance of the network including the

dHPC (factor 1), whereas inappropriate CS-US association is in accordance with LHbM/ LHbL inactivation and the consecutive disturbance of the network encompassing the CeA and the LA (factor 2). The c-Fos pattern in the LHb is also interesting regarding its potential role as a modulator of DA transmission during fear conditioning. Indeed, the part of the LHb that we defined as “rostral” includes the LHbMA subdivision described by Andres et al. (1999) which neurons send excitatory projection to VTA DA neurons (Metzger et al. 2019). In addition, neurons situated in the more caudal-lateral region of the LHb send excitatory projection on GABA neurons of the RMTg (Petzel et al. 2017) which in turn inhibit VTA DA neurons activity (Brown et al. 2017). The strong activation of these LHb subregions, where hM4(Gi) receptors were abundantly expressed, suggests that the observed deficits could be the consequence of the disturbance of the fine-tuning of DA transmission exert by the LHb during conditioning. Interestingly, infusion of D1-like receptor antagonist during training was shown to reduce trace CS fear memory when infused in the prelimbic region of the mPFC (Runyan and Dash, 2004), and to increase contextual fear when infused in the shell part of the nucleus accumbens (Albrechet-Souza et al., 2013). One can, therefore, postulate that the enhanced CS- and reduced contextual memory seen in hM4-CNO rats is consecutive to an increased DA flow within these two brain regions, due to the disinhibition of DA transmission by LHb inactivation, as suggested by previous work (Lecourtier et al. 2008). However, the fact that D1-like antagonist infusion during conditioning in other VTA DA targets, i.e. the dorsomedial PFC (Stubbendorff et al. 2019), dHPC and BL (Heath et al. 2015) reduces contextual fear rather than increasing it, renders difficult to explain our results just by global disinhibition of the DA transmission. Moreover, D1-like antagonism and agonism were both shown to decrease contextual fear when infused in the LHb during conditioning (Chan et al. 2017). Thus, if numerous evidence pointed to the involvement of DA in various target regions of VTA neurons during fear conditioning where and how perturbations of DA signaling upon LHb inactivation modulates fear learning remains to be explored. Besides DA, another key implication of the LHb during conditioning could be to contribute to the proper modulation of the hypothalamic-pituitary-adrenal axis response. Kaouane et al. 2012 have shown that acute restraint stress and postconditioning intrahippocampal, or systemic, corticosterone (CORT) injections, induce memory impairments reminiscent with those observed in the current study in hM4-CNO rats. Indeed, animals conditioned with an explicitly CS-US unpaired training procedure disregarded the context as the correct predictor of a footshock and showed fear of the unpredictable cue. We have previously shown that the blood CORT response to a stressful experience was higher in rats with inactivated LHb (Mathis et al. 2018); one can, therefore, hypothesize that a too large CORT release during conditioning has contributed to the memory impairments observed in hM4-CNO rats. Such a CORT-mediated effect would be congruent with the general role of the LHb in the modulation of fear learning. In summary, we have shown that LHb inactivation during the conditioning phase of a trace fear conditioning paradigm led to contextual memory deficits along with an enhanced response to a discrete cue. This study improves our understanding of the neuroanatomical bases of fear memory by showing that the LHb is crucially involved in the selection of the more relevant cue predicting a danger.

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