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The reuniens and rhomboid nuclei are necessary for contextual fear memory persistence in rats

Etienne Quet^{1,2} · Monique Majchrzak^{1,2} · Brigitte Cosquer^{1,2} · Thomas Morvan^{1,2} · Mathieu Wolff^{3,4} · Jean-Christophe Cassel^{1,2} · Anne Pereira de Vasconcelos^{1,2} · Aline Stéphan^{1,2}

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Abstract

Memory persistence refers to the process by which a temporary, labile memory is transformed into a stable and long-lasting state. This process involves a reorganization of brain networks at systems level, which requires functional interactions between the hippocampus (HP) and medial prefrontal cortex (mPFC). The reuniens (Re) and rhomboid (Rh) nuclei of the ventral midline thalamus are bidirectionally connected with both regions, and we previously demonstrated their crucial role in spatial memory persistence. We now investigated, in male rats, whether specific manipulations of ReRh activity also affected contextual and cued fear memory persistence. We showed that the permanent ReRh lesion impaired remote, but not recent contextual fear memory. Tone-cued recent and remote fear memory were spared by the lesion. In intact rats, acute chemogenetic ReRh inhibition conducted before recall of either recent or remote contextual fear memories produced no effect, indicating that the ReRh nuclei are not required for retrieval of such memories. This was also suggested by a functional cellular imaging approach, as retrieval did not alter *c-fos* expression in the ReRh. Collectively, these data are compatible with a role for the ReRh in 'off-line' consolidation of a contextual fear memory and support the crucial importance of ventral midline thalamic nuclei in systems consolidation of memories.

Keywords Fear memory · Hippocampus · Medial prefrontal cortex · Systems consolidation · Memory persistence · Ventral midline thalamus

Introduction

The persistence of a memory requires that encoded information gradually stabilize, whereby becoming resistant to interference. Systems consolidation designates the progressive

reorganization of the brain structures that support memory persistence (Squire and Alvarez 1995; Frankland and Bontempo 2005). According to the standard theory of systems consolidation (Marr 1971), the initial information encoded with a contribution of the neocortex is integrated by the hippocampus (HP). After learning, off-line hippocampo-cortical reactivations occurring during sleep and quiet wakefulness reinforce cortico-cortical connections (Born and Wilhelm, 2012). As a result, memory becomes more reliant on cortical circuits and less on the hippocampus proper.

The reuniens and rhomboid nuclei (ReRh) of the ventral midline thalamus have dense and reciprocal anatomical connections with the HP (CA1 and subiculum) and the medial prefrontal cortex (mPFC). The ReRh mainly project to the ventral HP or the mPFC (infralimbic, prelimbic and anterior cingulate cortex) (McKenna and Vertes 2004; Vertes et al. 2006; Hoover and Vertes 2012; Varela et al. 2014). In addition, 5–10% of Re neurons send collaterals in both structures (Hoover and Vertes 2012; Varela et al. 2014). Electrophysiological studies indicate that the Re nucleus exerts direct

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excitatory and indirect inhibitory actions on HP (CA1) and mPFC. Furthermore, the Re nucleus plays a key role in the hippocampo-cortical synchronization of delta, gamma and/or theta oscillations at work during various cognitive tasks (rev Dolleman-van-der-Weel et al. 2019). Considering that the HP directly projects to the mPFC, and that there are no direct return projections from the mPFC to the HP (Jay and Witter 1991; Hoover and Vertes, 2007), ReRh nuclei appear to be a key component of a hippocampo-cortical (mPFC) network supporting various aspects of memory functions (rev Cassel et al. 2013; Dolleman-van-der-Weel et al. 2019). We previously found that the permanent, fiber-sparing lesion of these thalamic nuclei supported their contribution to the persistence of spatial memory, but neither to its encoding nor to its retrieval (Loureiro et al. 2012). The ReRh is also important for fear memory reconsolidation (Sierra et al. 2017) and was suggested to act as a hub between the HP and mPFC for remote fear memory formation and/or expression (Vetere et al. 2017).

In the present study, we examined the role of the ReRh in the consolidation of HP-dependent and HP-independent fear memories at systems level. To this end, we assessed in rats the impact of a pre-acquisition, fiber-sparing excitotoxic lesion of the ReRh on contextual and cued conditioned fear, at either recent (1d) or remote (25d) time points. We then investigated whether the contribution of the ReRh to remote contextual fear memory was online (during information recall) using both ReRh chemogenetic inhibition and *c-fos* expression imaging. Altogether, our data are compatible with an off-line contribution, most probably during systems level consolidation.

Materials and methods

Animals

The study conformed to the rules of the European Community Council Directive (2010/63/EU) and the French Agriculture Ministry. All approaches have been validated by the ethical committee of the University of Strasbourg (CREMEAS—authorizations #5822-2016062214582106 and #13261-2018012918394046).

All experiments used Long–Evans rats (Janvier Labs, Le Genest-Saint-Isle, France) weighing 250 g at their arrival at the laboratory. Animals were housed two or three per cage in quiet facilities, under a 12 h light/dark cycle (light on at 7:00 A.M.) with food and water ad libitum, controlled temperature, and a hygrometry of about 55%. Before any experimental manipulation (surgery or behavioral training), rats were individually handled for 2 min/day over five consecutive days.

Experiment 1

The first experiment aimed to examine the effect of a permanent ReRh lesion on cued and contextual fear memory tested at both recent (1d) and remote (25d) time points.

ReRh lesion

For permanent fiber-sparing excitotoxic lesions of the ReRh, subjects were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and secured in a stereotactic frame. This anesthetic was used instead of ketamine to avoid the latter's interaction with *N*-methyl-D-aspartate (NMDA) receptors. As previously described (Loureiro et al. 2012), neurotoxic lesions targeting the ventral midline thalamus (ReRh) were made using slow microinfusions (over 5 min) of 0.12 M NMDA (0.1 μ l/site; Sigma), dissolved in phosphate-buffered saline (PBS), via an infusion needle (0.28 mm in diameter) connected to a motorized infusion pump. After leaving the needle in situ for an additional 5 min to ensure diffusion of NMDA into the target structure, the needle was slowly retracted. Infusion sites were located as follows (in mm): AP = – 1.5; – 2.1 and – 2.7 (from bregma), DV = – 7.0; – 7.1 and – 7.2 (from skull), respectively, ML = – 1.9 (from midline of the sagittal sinus), using an ML angle of 15° (Paxinos and Watson, 2007). The sham-operated controls (Sham) were infused with PBS instead of NMDA at the same coordinates. All rats that underwent surgery were allowed to recover under a warm lamp for 20–30 min before being placed back into their home cage. They were given a 2-week rest period before the start of behavioral testing.

Cued and contextual fear conditioning

A complete description of the testing apparatus and experimental procedure can be found in Majchrzak et al. (2006). Conditioning and testing took place in six identical conditioning chambers (25 \times 27 \times 18 cm) made of transparent plastic with a transparent ceiling and placed in ventilated (background noise between 65.7 and 70.2 dB) light- and sound-attenuated boxes (57 \times 38 \times 38 cm, Campden Instruments). An illumination of 6 lx was maintained by a bulb through a frosted plastic plate. A camera (MCT-210 MS, OptoVision, Toulouse, France) was fitted inside each box, above the center of the chamber, such as to monitor the entire chamber. The grid floor of each chamber consisted of parallel 0.3 cm-diameter stainless-steel bars spaced 0.8 cm apart. A sawdust tray was placed under the grid floor. Tone fear test was conducted in the same chambers modified to define a different context by changing tactile, olfactory and visual

cues. Tone and shock delivery were controlled by a computerized interface (Med-PC, Med Associates, St Albans, VT). Automatic freezing measurements were performed as described in detail by Marchand et al. (2007). Briefly, video signals were sent to a computer equipped with a Scion LG3 video capture card (Scion Corporation, Frederick, MD) via two Quad-type multiplexers (Computar QSMX-II). Data acquisition was carried out by a script written under the ‘‘Scion Image’’ software, which allowed the monitoring of all chambers at a sampling rate of 1 Hz. The analysis of freezing behavior was done with a set of procedures written under Excel[®] Visual Basic[®], which allowed the computation of the percentage of freezing time over blocks of selected duration.

Fear conditioning procedure

The conditioning session consisted of five tone presentations (15 s, 4000 Hz, 10 dB above background) associated with one foot shock (0.6 mA before scrambling, 0.8 s) delivered at the offset of the tone. The first tone was presented 3 min after the placement of the rat in the chamber, and then at variable time intervals (452 ± 96 s). The total session duration was 38 min. The context test session took place either on the following day (to tax recent memory) or after a 25-day delay (to tax remote memory). Conditioned freezing to the context was assessed by placing rats in the same conditioning chamber for a 10-min test session. Twenty-four hours later (d2 or d26), a 10-min extinction session was conducted in the modified chamber in prevision of the following tone fear test. The next day (d3 or d27), the tone fear test session was performed by placing rats in the modified chamber and the tone was presented five times, the first presentation 2 min after the placement of the rat in the chamber and then at a fixed time interval (255 s) (Fig. 1b).

Experiment 2

The second experiment aimed to study the effect of temporary inactivation of the ReRh during recent (1d) and remote (25d) contextual fear memory retrieval. The DREADD approach was then validated in the double-H maze using a cognitive flexibility task known to be highly dependent upon the integrity of the ReRh nuclei (Cholvin et al. 2013).

DREADD inactivation of the ReRh

For DREADD viral injections in the ReRh, subjects were anesthetized with ketamine (98 mg/kg)–xylazine (13 mg/kg, i.p.). $2 \times 0.4 \mu\text{l}$ of 7.3×10^9 genomic copies/ μl of AAV8-Camk2 α -hM4Di-mCherry (HM4, Viral Vector Production Unit, Spain) were injected in the ReRh at the

following coordinates: AP = -1.6 and -2.6 (from bregma), DV = -7.1 and -7.2 (from skull), ML = -1.9 (from midline of the sagittal sinus), using a ML angle of 15° (Paxinos and Watson 2007). The sham-operated controls (Sham) were infused with phosphate-buffered saline instead of virus solution at the same coordinates. Rats were given a 3-week rest period before the start of behavioral testing, allowing at least 1 month of virus expression before the first CNO injection.

Chemogenetic inactivation of the ReRh was performed by intra-peritoneal injection of clozapine-*N*-oxide (CNO 1 mg/kg, Enzo life sciences) 45 min before behavioral testing. For DREADD experiments, all animals (HM4 and Sham) received a CNO injection to check for any non-specific effect of this ligand or derived product clozapine (Gomez et al. 2017).

Contextual fear conditioning procedure

The same apparatus as in experiment 1 was used for contextual fear conditioning in experiment 2. The conditioning procedure was previously described in Bousiges et al. (2013). The conditioning session consisted of three footshocks (0.6 mA before scrambling, 0.8 s) that were delivered 180, 240, and 360 s after the start of the conditioning session (total session duration, 8 min). The context test session took place either on the following day (recent memory) or after a 25-day delay (remote memory) (Fig. 3a). Conditioned freezing to the context was assessed by placing rats in the same conditioning chamber for a 10-min test session.

The double-H maze task

A detailed description of the testing apparatus and experimental procedure (illustrated in Fig. 4a) can be found in the publication by Cholvin et al. (2013).

Apparatus

Briefly, the double-H maze is made of three parallel running arms, 160 cm long \times 20 cm wide, connected to each other at the level of their center by a 160 cm long \times 20 cm wide corridor. By convention, the two opposing arms in the middle are designed as north (N) and south (S), respectively. The extremities of both other pairs of side arms correspond to the four potential target locations. They are termed northwest (NW), northeast (NE), southwest (SW), and southeast (SE) hereafter.

Training protocol

The double-H was filled with water (21 °C) made opaque by addition of powdered milk (1.5 g/L). A platform, 11 cm diameter, was immersed 1 cm beneath the water surface at

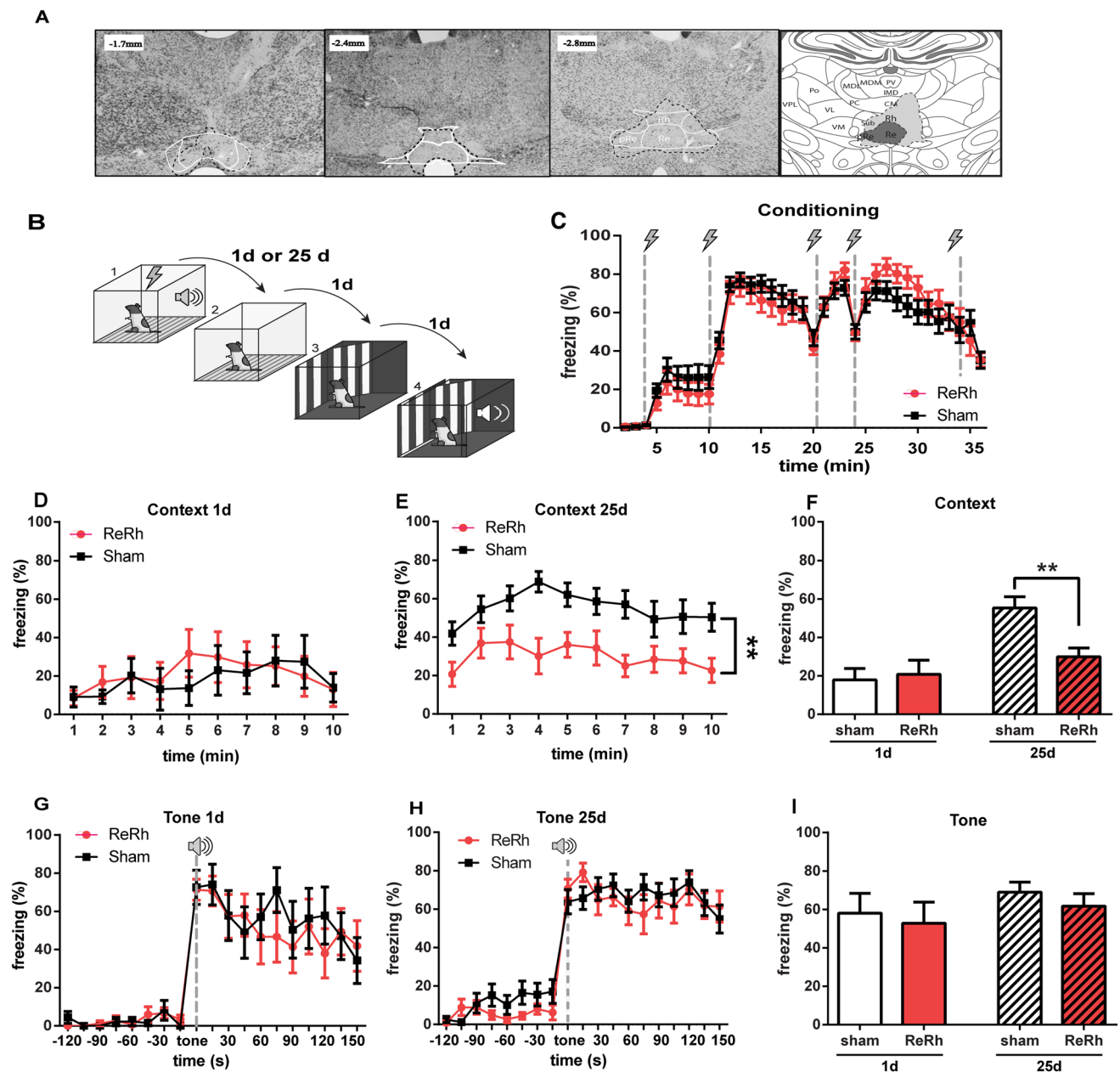


Fig. 1 Effect of ReRh lesion on contextual and cued fear memory (Experiment 1). **a** Photomicrographs showing typical examples of NeuN-immunostained brain sections from a rat with ReRh lesion taken at three antero-posterior levels (as for all the rats) as well as schematic representations of the smallest (dark gray) and largest (light gray) lesion of the ventral midline thalamic nuclei (Re and Rh). **b** Schematic representation of the fear memory protocol: rats underwent one fear conditioning session (1) either 1 or 25 days before contextual fear memory test (2). The extinction session (3) of non-specific contextual fear was conducted on the next day. The tone test (4) took place on the following day. **c** Freezing behavior across fear conditioning session (grouped 1d and 25d rats). Dotted lines indicate each tone-foot shock presentation. **d** Percentage of freezing during the recent (1d) contextual fear test. **e** Percentage of freezing during the remote (25d) contextual fear test. **f** Mean percentage of freezing

during recent (1d) and remote (25d) contextual fear memory tests. **g** Percentage of freezing during the first tone presentation of the recent (3d) cued fear memory test. Dotted line indicates tone presentation. **h** Percentage of freezing during the first tone presentation of the remote (27d) cued fear memory test. Dotted line indicates tone presentation. **i** Mean percentage of freezing during the 2-min period following the first two tone presentations during recent (3d) and remote (27d) cued fear memory tests. $**p < 0.01$, statistically significant difference (Newman-Keuls). *CM* central medial thalamic nucleus, *IMD* intermediodorsal thalamic nucleus, *MDL* lateral mediodorsal thalamic nucleus, *MDM* medial mediodorsal thalamic nucleus, *PC* paracentral thalamic nucleus, *Po* posterior thalamic nucleus, *pRe* perireuniens thalamic nucleus, *PV* paraventricular thalamic nucleus, *Sub* submedius thalamic nucleus, *VL* ventrolateral thalamic nucleus, *VM* ventromedial thalamic nucleus, *VPL* ventral posterolateral nucleus

the extremity of the NE arm. For each rat, the task consisted of learning to swim from the start point—which was changed randomly between S and N—to the escape platform. A first day of pre-training habituated rats to the water and testing device. Over the four following training days, rats were given four daily trials separated from each other by a 10 s interval (Fig. 4a). They were released in the maze either from the N or the S arm in a randomized order (e.g., S,N,S,N on day 1; S,N,N,S on day 2; N,S,N,S on day 3, and S,N,S,N on day 4). When the rats were released from the N, the S arm was closed by a transparent guillotine door to prevent any entry, and vice versa. Each trial, whatever the protocol, lasted for a maximum of 60 s. When a rat did not reach the platform within this delay, it was gently guided to the platform by the experimenter. The variables recorded were the distance and latency to reach the target arm as well as the swim velocity.

Probe trial

Two probe trials were given: one 24 h after the first 2 days of training, and another 24 h after two additional days of training (Fig. 4a). For both probe trials, the platform was removed from the maze. The probe trial duration was 60 s. All rats were released from the SW arm, with the entry of NW arm closed by a guillotine door. This procedure (1) prevented the behavior displayed during maze habituation to be reproduced (i.e., swim directly from SW to NW), (2) made one of the learned successive turn sequences impossible (i.e., the left–left sequence), (3) did not disable the right–left sequence, and (4) allowed rats to shift from a strategy based on the right–left turn sequence to a spatial approach of the task (search in target arm), and to do so either directly or as a consequence of negative feedback (corresponding to entering the N arm after successive right and left turns). Whether the shift to a spatial strategy was immediate or not, evidence for searching the platform at the correct place can be interpreted as the result of a strategy shift. The variables recorded and analyzed were the time spent in the former target arm (i.e., NE, termed “place arm” hereafter) and the time spent in the arm to which successive right–left turns were leading (i.e., N, termed “response arm” hereafter).

Histological verifications (experiments 1 and 2)

All rats were subjected to a lethal dose of pentobarbital (200 mg/kg, i.p.) and then perfused transcardially with 4% paraformaldehyde (PFA). Brains were removed and transferred to a 20% sucrose solution for 72 h at 4 °C before being snap frozen (isopentane, – 40 °C) and stored at – 80 °C. Free-floating coronal sections (40 µm) were cut using a cryostat (Microm HM560, Thermo Scientific).

NeuN immunostaining

To complete the histological characterization of the ReRh lesions (experiment 1), immunolabeling of the NeuN protein was performed on brain sections evenly distributed along the entire rostro-caudal extent of the ReRh. The protocol was similar to the one used for c-Fos immunohistochemistry (see below paragraph), using a mouse NeuN antibody (1:2000, ref MAB377; Millipore) as primary antibody, and a biotinylated anti-mouse horse antibody (1:500; Vector Laboratories) as secondary antibody (see Loureiro et al. 2012).

Viral infection and terminal sites visualization

To evaluate the viral infection in ReRh, brain sections from DREADD-injected rats (experiment 2) were rinsed 3 × 10 min in PBS before being mounted using Mowiol mounting medium (Sigma-Aldrich). Direct fluorescence of the AAV-encoded mCherry was visualized using a Nanozoomer (Model S60, Hamamatsu). The fibers and terminal sites of ReRh-infected cells were observed after an amplification of mCherry signals and a counterstaining with calretinin performed on sections of frontal and hippocampal regions. The immunostaining protocol was similar to the one used for c-Fos immunohistochemistry. A DsRed polyclonal antibody (1/1000, Takara) was used to amplify the mCherry fluorescence and, for counterstaining, a mouse calretinin antibody (1:8000, rabbit, Swant) was used as the primary antibody, and an Alexa A488 anti-mouse antibody (1:1000; Invitrogen) as the secondary one.

Quantification of the lesion/virus infection (experiments 1 and 2)

Serial sections (40 µm) throughout the midline thalamus were cut using a cryostat to assess the lesion/infection placement and extent. Lesions/infections were drawn using the relevant plates of the rat brain atlas (Paxinos and Watson 2007) and replicated on electronic copies of the atlas. Automated pixel counts of the thalamic nuclei in the target regions were used to estimate lesion/infection extent. Acceptable lesions/infections were defined as having > 50% damage/infection to the ventral midline thalamus (Reuniens, Rhomboid and peri-Reuniens nuclei combined) with at least 15% damage/infection to each of the nuclei (Re, Rh, right pRe and left pRe) to prevent the inclusion of asymmetric lesion/infection (Loureiro et al. 2012).

Experiment 3

The third experiment aimed to study ReRh neuronal activity during recent (1d) and remote (25d) contextual fear memory retrieval by quantification of the immediate early gene *c-fos* expression.

Experimental groups and contextual fear conditioning

Three groups of unoperated rats were used: “conditioned” rats underwent the same contextual fear conditioning procedure as in experiment 2; “not-conditioned” control rats underwent the same procedure except that no foot shock was delivered during the conditioning session; and “home cage” control rats remained in their home cage throughout the entire procedure. Within each group, half the rats were tested after a 1-day delay and the other half after a 25-day delay (Fig. 5a).

c-Fos immunohistochemistry and quantification

Ninety minutes after behavioral test completion (Fig. 5a), rats were subjected to a lethal dose of pentobarbital (200 mg/kg, i.p.) and perfused transcardially as for experiments 1 and 2. The following protocol of immunohistochemistry has been previously described in Lopez et al. (2012). Briefly, sections were rinsed three times during 10 min in PBS before being soaked for 1 h in 5% normal donkey serum in PBS containing 0.5% Triton X-100. They were subsequently transferred into the primary anti-Fos rabbit polyclonal antibody (1:4000; Santa Cruz Technology) solution overnight at room temperature. Then, the sections were soaked in a buffer solution containing biotinylated goat anti-rabbit secondary antibody (1:500, Biotin-SP-conjugated affiniPure; Jackson ImmunoResearch). Staining was revealed with the avidin–biotin peroxidase method (Vectastain ABC kit; Vector Laboratories) coupled to diaminobenzidine.

The quantitative analyses of c-Fos-positive nuclei were performed in the ventral midline nuclei (Re, Rh) (– 1.3 to – 3.3 mm from bregma, Paxinos and Watson 2007) on brain sections along their entire rostro-caudal extent. Stained sections were photographed ($\times 10$ objective lens). The same intensity of light as well as the same parameters for exposure time of the digital camera was used for all sections. Using the Fiji software (Schindelin et al. 2012), the regions of interest were delimited and c-Fos-positive neurons counted using a threshold value that kept all immune-labeled positive cells but no background.

Statistical analysis (all experiments)

Unless otherwise specified and depending on the experiment, data analyses used Student's *t* tests or two-way ANOVAs, with repeated measures where appropriate. Likewise, where appropriate, these analyses were completed by multiple comparisons using the Newman–Keuls multiple range test. The time spent in the arms during the double-H maze probe trials was also compared to chance using a one-sample *t* test. Chance level was 8.2 s in the double-H [(surface of

one arm/accessible surface of the maze)/60 s]. Values of $p < 0.05$ were considered significant. A χ^2 analysis was used to compare categorical variables in the double-H maze.

Results

ReRh lesion specifically alters expression of a remote contextual fear memory (Experiment 1)

Acceptable lesions were defined as having $> 50\%$ damage to the ventral midline thalamus (Re, Rh and peri-Re nuclei combined) with at least 15% damage to each of the nuclei to avoid asymmetrical lesions (see methods). Damage to thalamic structures other than ReRh was generally minimal or modest. Figure 1a shows typical examples of an ReRh lesion illustrated at three antero-posterior levels as well as an example of the largest and smallest ReRh lesions observed in rats that were included in the behavioral analyses. Final sample sizes were as follows: 1d groups, $n_{\text{ReRh}} = 10$, $n_{\text{Sham}} = 9$; 25d groups, $n_{\text{ReRh}} = 15$, $n_{\text{Sham}} = 22$.

No significant differences in the extent of the ReRh lesion were found between the 1d- and 25d-delay groups ($F_{(1,46)} = 0.16$, $p = 0.69$). In the 10 ReRh rats of the 1d-delay group, there was a median of 91% damage to the Re, 74% to the left pRe, 83% to the right pRe and 79% damage to the Rh nuclei. In the 15 ReRh rats of the 25d-delay group, there was a median of 85% damage to the Re, 54% to the left pRe, 66% to the right pRe, and 62% damage to the Rh. Damage to thalamic structures other than ReRh, including midline nuclei, was generally minimal to modest. The median damage was always $< 10\%$ in both groups for each of the following thalamic nuclei: submedius, central medial, intermediodorsal, and mediodorsal (with a maximum value of 17% and 15% for the submedius nucleus in the 1d- and 25d-delay groups, respectively).

To investigate the role of the ventral midline thalamus in fear memory processing, we tested whether a pre-acquisition excitotoxic lesion of the ReRh disrupted the formation and/or expression of contextual- and cued fear memory. An extinction session of non-specific contextual fear was conducted on the next day (d2 or d26), and a tone-cued fear test the day after (d3 or d27) (Fig. 1b).

During conditioning, freezing responses developed in all groups to reach similar levels of freezing and showed a significant effect of Time ($F_{(34,1836)} = 71.78$, $p < 0.0001$), but no effect of the ReRh lesion (Lesion, $F_{(1,54)} < 1.0$, $p = 0.98$; Lesion \times Time, $F < 1$, $p = 0.98$), suggesting that the lesion did not modify the acquisition of contextual fear memory, or foot-shock sensitivity (Fig. 1c).

The analysis of the percentage of freezing during contextual fear memory tests (Fig. 1f) showed no significant effect of the Lesion ($F_{(1,52)} = 2.947$, $p = 0.092$), but a

significant effect of the Delay ($F_{(1,52)} = 12.54$, $p = 0.0008$) with stronger fear responses for remote memory. Critically, the Delay \times Lesion interaction reached significance ($F_{(1,52)} = 4.61$, $p = 0.0365$), showing that ReRh lesion differently affected contextual fear recall as a function of post-conditioning delay, i.e., recent vs remote. The analyses indeed confirmed that freezing behavior was reduced by ReRh lesion for remote ($F_{(1,35)} = 4.12$, $p < 0.01$), but not recent memory ($F_{(1,17)} < 1.0$, $p = 0.77$) (Fig. 1d, e). Thus, the significant difference between the two groups at the 25d-delay points to a specific effect of the ReRh lesion on remote contextual fear memory could be due to alteration of consolidation and/or of remote memory recall processes.

During the cued fear memory test, freezing behavior was similar across groups and delays (Fig. 1i). There was no significant effect of the Group ($F_{(1,52)} < 1.0$, $p = 0.43$), the Delay ($F_{(1,52)} = 1.585$, $p = 0.21$), and no significant interaction between these factors ($F_{(1,52)} < 1.0$, $p = 0.90$). Responses to the first presentation of the tone at each delay are shown in Fig. 1g, h. The absence of effect of the ReRh lesion on cued fear regardless of the delay indicates that the ReRh are not necessary for cued fear memory encoding, consolidation and retrieval.

Altogether, these data suggest a specific implication of the ReRh in remote contextual fear memory expression. To examine whether the ReRh contributed to remote memory retrieval online process, we next examined the outcome of acute ReRh chemogenetic inhibition during recall.

Chemogenetic ReRh inactivation did not affect contextual fear memory retrieval (Experiment 2)

Viral infections were considered acceptable if they included $> 50\%$ of the ventral midline thalamus (Re, Rh and peri-Re nuclei combined) and at least 15% of each nucleus; these criteria were identical to the ones used for evaluation of excitotoxic lesions (see “Experiment 1” section). Figure 2a shows typical examples of ReRh viral infection at three antero-posterior level and the largest and smallest ReRh infections (at Bregma $- 2.2$ mm level, as example) that were included in this study. Final sample sizes were as follows: 1d groups, $n_{\text{ReRh}} = 17$, $n_{\text{Sham}} = 9$; 25d groups, $n_{\text{ReRh}} = 14$, $n_{\text{Sham}} = 9$. For the 31 HM4 rats that matched the inclusion criteria, there was a 73% median infection in the Re, 80% in the Rh, 49% in the left pRe and 44% in the right pRe. Interestingly and conveniently, the AAV8 with Camk2 α promotor did not produce any infection in the adjacent submedial thalamic nucleus. Infections were restricted to the targeted structures, except for some rats where small infections could be observed alongside the canulae tract through the centromedian nucleus of the thalamus (median: 13%).

In addition, Fig. 2b shows the labeled terminal sites of ReRh efferents in the HP, as well as in the mPFC. A dense

labeled band was observed in the dorsal (Fig. 2b) and ventral HP, the stratum lacunosum moleculare (slm) of CA1 specifically. Layers 1 and 5 of the perirhinal cortex were also densely labeled, as also the retrosplenial cortex and the periaqueductal gray path at a rostral level. More frontally (Fig. 2b), dense labeling concentrated in layers 1 and 5/6 of the ventral mPFC, mainly in the infralimbic and prelimbic cortex. There was also labeling at the level of the rhinal fissure of the agranular insular cortex and the accumbens nucleus. All these termination sites are in accordance with the strong connectivity between the ReRh nuclei and the aforementioned limbic regions (Vertes et al. 2006).

To investigate the role of the ventral midline thalamus in contextual fear memory retrieval, we tested whether a chemogenetic ReRh inactivation prior to testing disrupted the expression of contextual fear. In this experiment, both Sham and HM4 rats were conditioned as described in the methods and tested for contextual fear memory either 1d (recent memory) or 25d (remote memory) later, 45 min after CNO (1 mg/kg i.p.) injection (Fig. 3a).

During the conditioning session, virus injection (HM4) did not modify freezing behavior as compared to Sham rats (Fig. 3b, Time, $F_{(7,329)} = 300.8$, $p < 0.0001$; Group $F_{(1,47)} < 1.0$, $p = 0.44$); the interaction was not significant, Group \times Time, $F < 1$, $p = 0.94$).

During the test session, the analysis of the freezing behavior across the 10-min sessions (Fig. 3c, d) revealed no significant effect of the Group ($F_{(1,45)} < 1.0$, $p = 0.94$), of the Delay ($F_{(1,45)} = 2.207$, $p = 0.14$), and no interaction between these factors ($F_{(1,45)} < 1.0$, $p = 0.68$). Thus, ReRh chemogenetic inactivation during test session did not produce any deficit of contextual fear memory retrieval, whatever the post-conditioning delay.

DREADD validation in a cognitive flexibility task in the double-H maze (Experiment 2)

To ensure that chemogenetic inhibitions were sufficient to produce behavioral effects, we used another behavioral test assessing cognitive flexibility. This task was previously shown to depend upon ReRh integrity (Cholvin et al. 2013). Therefore, Sham and HM4 rats were trained and tested in the double-H maze (see protocol in Fig. 4a) 2 weeks after the contextual fear test.

During training, the distances swum before reaching the platform decreased over days ($F_{(3,51)} = 35.45$, $p < 0.0001$), indicating learning (Fig. 4b). There was no overall significant difference among the two groups, as attested by the absence of a significant Group effect ($F_{(1,17)} < 1.0$, $p = 0.5309$) and no interaction between the two factors ($F_{(3,51)} < 1.0$, $p = 0.82$). Final training performance levels (day 5) did not differ among groups. Analysis of latencies yielded similar conclusions (data not shown).

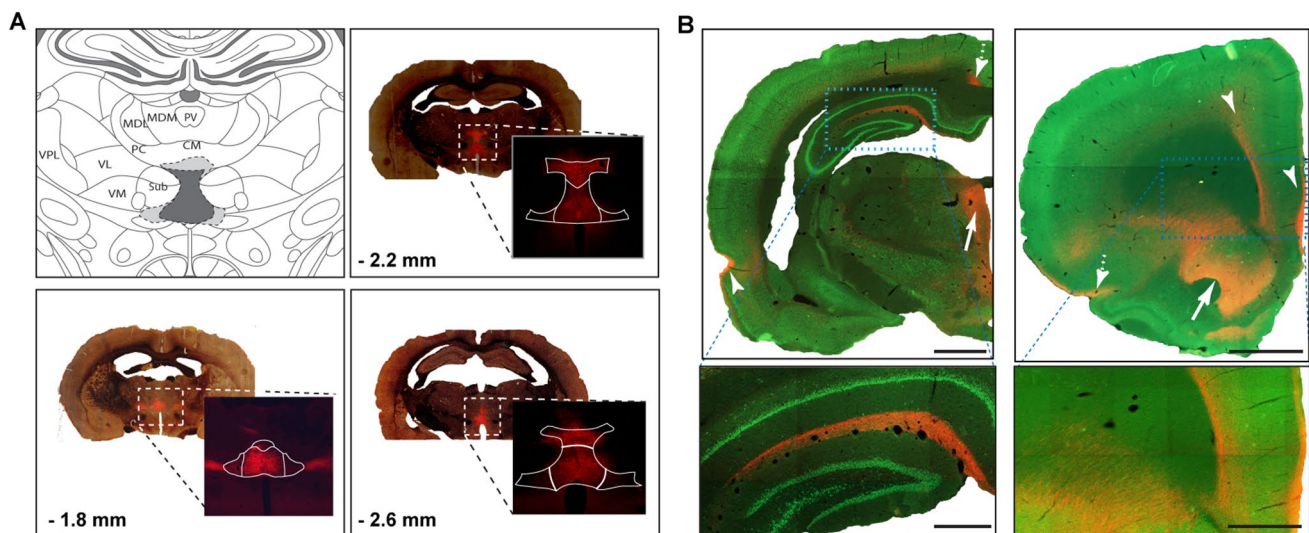


Fig. 2 Viral ReRh infection with AAV8-Camk2 α -hM4Di-mCherry (Experiment 2). **a** Top left panel, schematic representation of the smallest (dark gray) and largest (light gray) infection of the ReRh at the level β -2.2 mm (Paxinos and Watson 2007). Other panels A, photomicrographs showing typical examples of ReRh viral infections, visualized via mCherry fluorescence taken at three antero-posterior levels: β -1.8 mm; β -2.2 mm, β -2.6 mm. Note the visible tractus of the very thin needle in the Re on the bottom right A panel. **b** Photomicrographs showing main terminal sites of infected ReRh cells. Left, hippocampal level: we observed a dense band located in the stratum lacunosum moleculare (slm) of CA1. Layers 1 and 5 of the perirhinal cortex were also densely labeled (short arrow), as was the retrosplenial cortex (dotted arrow) and the periaqueductal gray path (full

arrow). Right, frontal level: dense labeling was concentrated in layers 1 and 5/6 of the mPFC (infralimbic and prelimbic cortex, short arrows). Notice also staining of the layer 1 of the agranular insular cortex, in the rhinal fissure (dotted arrow) and of the accumbens nucleus (full arrow). These projection sites from the ReRh nuclei were previously described by Vertes et al. (2006). Scale bar: 1000 μ m in top panels, 250 μ m in bottom panels. *CM* central medial thalamic nucleus, *IMD* interomediodorsal thalamic nucleus, *MDL* mediiodorsal thalamic nucleus lateral, *MDM* mediiodorsal thalamic nucleus medial, *PC* paracentral thalamic nucleus, *Po* posterior thalamic nucleus, *PV* paraventricular thalamic nucleus, *Sub* submedial thalamic nucleus, *VM* ventromedian thalamic nucleus, *VL* ventrolateral thalamic nucleus, *VPL* ventral posterolateral thalamic nucleus

During the first probe trial (Fig. 4b), the very first swim sequence consisted of a succession of right and left turns, leading 84% of rats to the N arm (response arm), whereas 16% directly swam to the former platform location (i.e., to the NE arm—place arm), with no significant difference among groups ($\chi^2 = 0.5322$, $p = 0.47$). During the second probe trial, the behavioral pattern was significantly different between Sham and HM4 rats ($\chi^2 = 5.143$, $p = 0.02$). Forty-four % of Sham rats, but no HM4 rats directly reached the former platform location (i.e., to the NE arm, place arm), while 90% of HM4 vs 56% of Sham rats first swam to the N arm (response arm).

In terms of relative amount of time spent in the place (NE) and response (N) arms, there was no Group difference during the first probe trial (NE arm: $t(17) < 1.0$, $p = 0.86$; N arm: $t(17) < 1.0$, $p = 0.59$, Probe 1). However, clear-cut differences emerged during the second probe trial after longer training (Fig. 4b, Probe 2). In Sham rats, the time spent in the NE arm was significantly above chance (one-sample t -test, $t(8) = 3.84$, $p = 0.005$), but it was not the case for HM4 rats ($t(9) < 1.0$, $p = 0.86$). Sham rats spent significantly more time than HM4 rats in the NE arm ($t(17) = 2.47$, $p = 0.02$). The analysis of the time spent in the response arm (N) showed no significant effect of inactivation, and

performance of Sham and HM4 rats did not differ from chance level (Sham: $t(8) = 1.541$, $p = 0.16$; HM4: $t(9) = 0$, $p > 0.99$; data not illustrated).

Thus, after four learning days, ReRh-inactivated rats failed to search for the platform as accurately as did Sham rats during the second probe trial, indicating a loss of flexibility in a spatial navigation task. Thus, the DREADD approach produced a behavioral deficit in a ReRh-dependent task, consistent with previous muscimol-induced ReRh inhibition (Cholvin et al. 2013).

Contextual fear memory retrieval did not induce *c-fos* expression in the ReRh (Experiment 3)

To confirm the inactivation data indicating an absence of implication of the ventral midline thalamus in contextual fear memory retrieval, we analyzed the immediate early gene *c-fos* expression following the probe test. Rats were either conditioned as described in the methods, or not conditioned (no foot-shock during the conditioning session) before being tested either 1d or 25d later, and killed 90 min after the fear memory test. A separate group of rats remained in the home cage during the entire procedure (HC). Sample sizes were

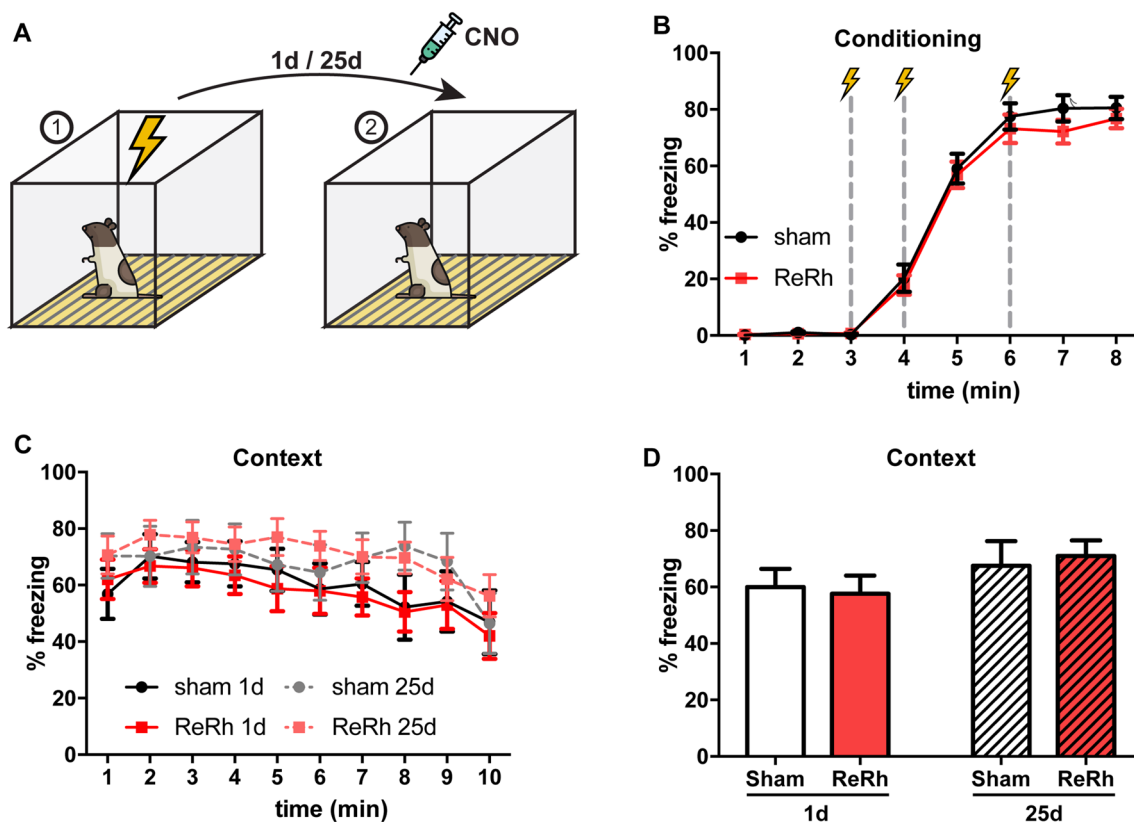


Fig. 3 DREADD inactivation of the ReRh during contextual fear memory retrieval (Experiment 2). **a** Schematic representation of the contextual fear memory protocol. Rats underwent one fear conditioning session (1) 1d or 25d before the contextual fear memory test (2). Rats were injected with CNO 45 min prior to the beginning of the memory test. **b** Percentage of freezing behavior across fear condition-

ing sessions (grouped 1d and 25d rats). Dotted lines indicate the three foot shocks. **c** Percentage of freezing behavior during recent (1d) and remote (25d) contextual fear memory tests. **d** Mean percentage of freezing during recent (1d) and remote (25d) contextual fear memory tests

as follows: 1d groups, $n_{\text{Cond}}=8$, $n_{\text{NotCond}}=8$, $n_{\text{HC}}=4$, 25d groups, $n_{\text{Cond}}=8$, $n_{\text{NotCond}}=7$, $n_{\text{HC}}=4$.

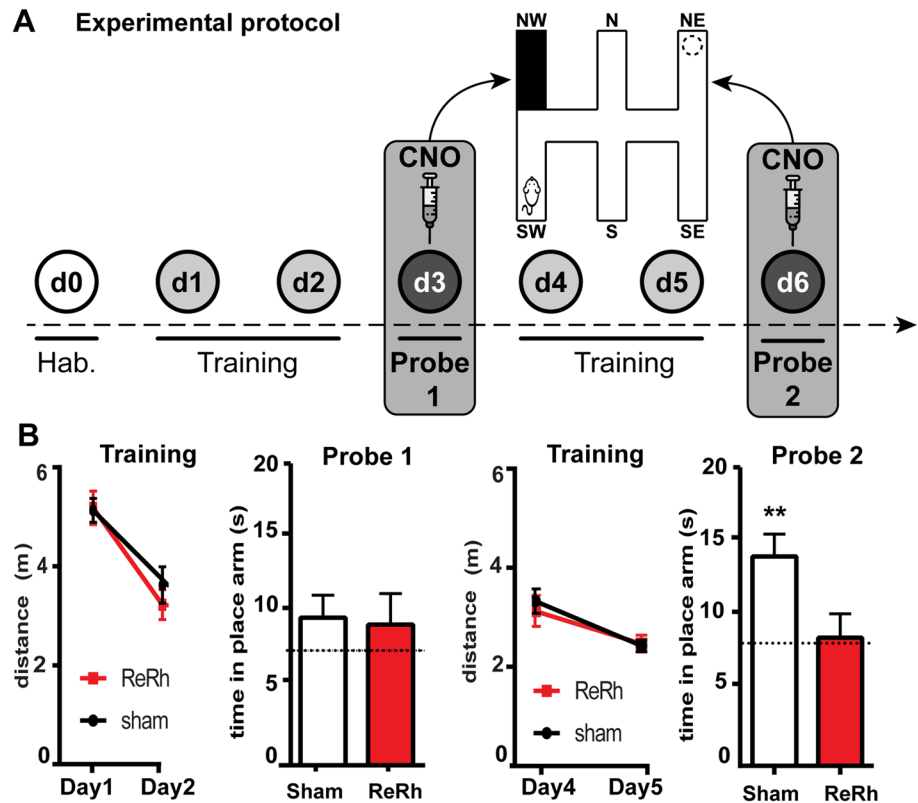
As expected, not-conditioned rats did not exhibit any freezing behavior during exposure to the context and test sessions. As expected, conditioned rats showed high freezing behavior during the memory tests, regardless of the delay (Fig. 5b, c). ANOVA of *c-fos* expression in the Re (Fig. 5e) and Rh nuclei (Fig. 5f) showed a significant effect of the Group (Re: $F_{(2,33)}=3.267$, $p=0.0507$; Rh: $F_{(2,33)}=5.241$, $p=0.0105$), a significant effect of the Delay, but only for the Re ($F_{(1,33)}=7.803$, $p=0.009$; Rh nucleus: $F_{(1,33)}=2.297$, $p=0.14$), and no interaction between both factors (Re: $F_{(2,33)}<1.0$, $p=0.95$; Rh: $F_{(2,33)}=1.485$, $p=0.24$). Post hoc Newman Keuls analysis showed that the Group effect was due to a significantly lower *c-fos* expression in the home cage (HC) rats as compared to not-conditioned rats (Re: $p<0.05$; Rh, $p<0.01$); it can be noticed that the difference was close to significance when compared with the conditioned groups (Re, $p=0.061$; Rh, $p=0.065$). In the Re nucleus only, the Delay effect reflected the significant increase in *c-fos* expression at 25-d vs 1-d delay. Finally,

post hoc analysis showed no significant difference in the number of *c-Fos*-positive cells between conditioned and not-conditioned rats, regardless of the delay and structure. Figure 5d shows typical examples of *c-fos* immunostaining. This result consolidates the absence of implication of the ReRh in contextual fear memory retrieval, in line with DREADD inactivation data.

Discussion

The present study examined the role of ReRh in fear memory persistence. A specific deficit was found for remote contextual fear memory, not for a recent one after ReRh lesion, whereas cued fear memory was not affected by the lesion, whether recent or remote. Chemogenetic ReRh inactivation had no effect on the retrieval of recent and remote contextual fear memory, but produced deficits in a strategy-shifting task known to recruit ReRh nuclei (Cholvin et al. 2013). Furthermore, *c-fos* expression in the ReRh was not affected during retrieval of contextual

Fig. 4 DREADD validation in a cognitive flexibility task using the double-H maze (Experiment 2). **a** Schematic representation of the experimental procedure. Rats underwent 1 day of habituation (day 0) before being trained for two consecutive days. A first probe trial occurred on day 3. Rats were then trained for two additional days before being tested in a second probe trial on day 6. Rats were injected with CNO 45 min prior to each probe trial. **b** Plots 1 and 3 show the distance traveled by the rats to reach the platform during the 4 days of training. Plots 2 and 4 show the time spent by the rats in the “place” arm (NE) during the two probe trials. Dotted line indicates chance level. $**p < 0.01$ vs chance



memory, whatever the post-conditioning delay. Taken together, these results indicate a specific implication of the ReRh thalamic nuclei in contextual (not cued) fear memory persistence in rats. They are also in line with the idea that ReRh nuclei might be key structures mediating off-line systems consolidation processes of hippocampus-dependent memories, as contextual fear memory.

ReRh in cued fear memory

Our experiments showed no effect of the ReRh lesion on cued fear memory, whatever the delay between the conditioning session and the tone test. Similarly, Ramanathan et al. (2018a) showed, in the rat, no effect of ReRh inactivation on the acquisition or expression of auditory fear conditioning at a recent time point. Likewise, Xu and Südhof (2013) had previously shown that Re neuronal silencing by TetTox before or after conditioning had no impact on cued fear memory. Cued fear conditioning as well as recent and remote retrieval engage the amygdala and does not require the HP (Phillips and LeDoux 1992; Bergstrom 2016). Thus, our data confirm that ReRh nuclei are not involved in such amygdala-dependent learning and memory processing.

ReRh in recent contextual fear memory

The importance of ReRh in contextual fear conditioning is in line with previous data showing the implication of these nuclei in HP-dependent processes (Loureiro et al. 2012; Hallock et al. 2016). Combining various approaches of transient ReRh inactivation, previous studies have shown disruptive effects of pre- or immediately post-conditioning ReRh inactivation on the retrieval of a recent contextual fear memory (Xu and Südhof 2013; Ramanathan et al. 2018b; Troyner et al. 2018). Indeed, these studies demonstrated that optogenetic or pharmacological inactivation of the Re nucleus during encoding caused an overgeneralization of contextual fear memory without significantly altering contextual fear recall in the original conditioning context at a 1 day delay. These studies showed that the Re nucleus is a key member of a brain network controlling fear memory specificity and generalization, but is not crucial for memorizing and recalling a precise contextual memory. Our results, showing no effect of ReRh permanent pre-conditioning lesion on recent contextual fear memory confirm these data. They are also in line with our previous report on acquisition and recent recall of a spatial memory (Loureiro et al. 2012).

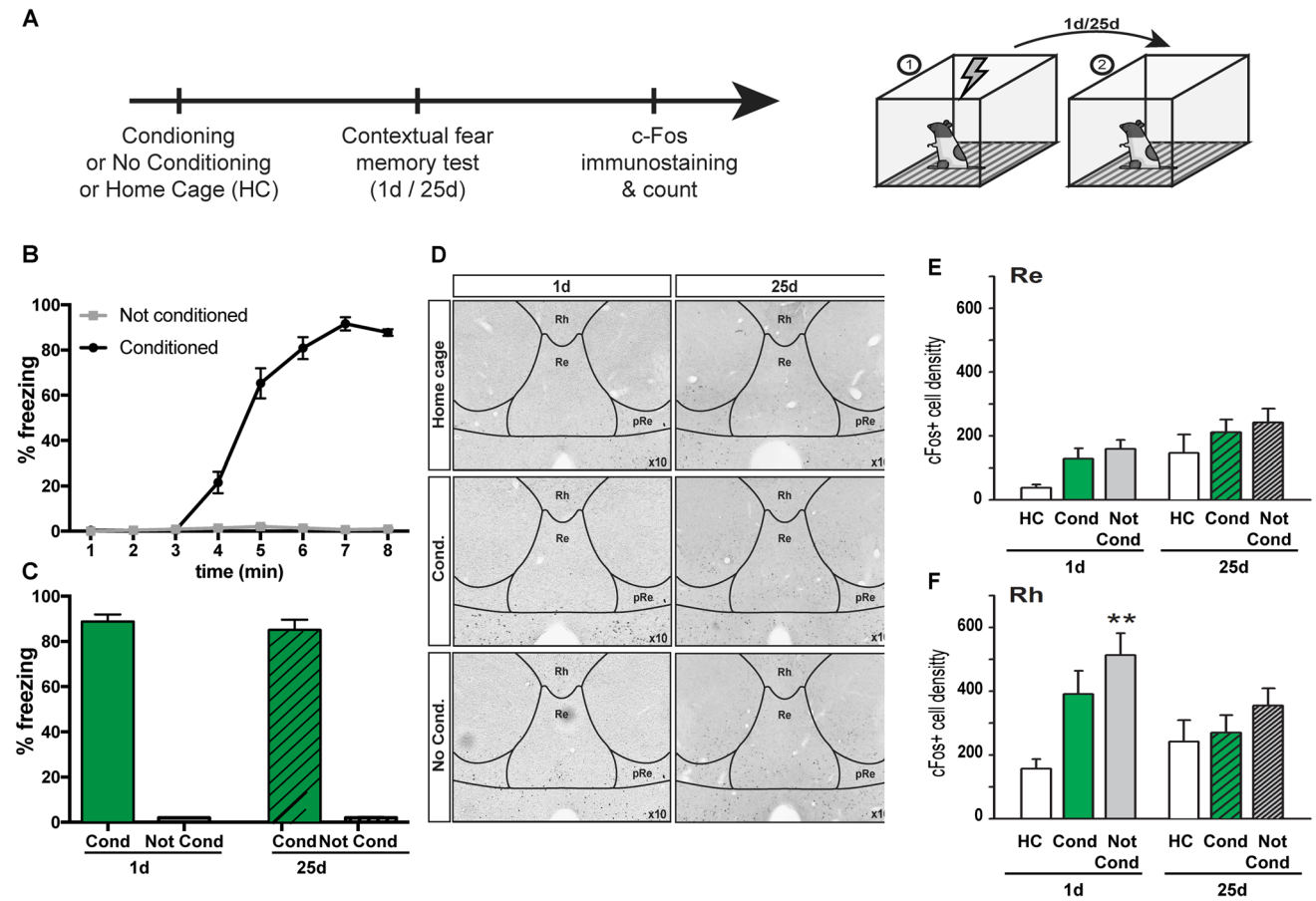


Fig. 5 *c-fos* expression in the ReRh following contextual fear memory retrieval (Experiment 3). **a** Schematic representation of the experimental procedure. **b** Freezing behavior during the conditioning sessions (grouped 1d and 25d rats). **c** Mean percentage of freezing during recent (1d) and remote (25d) contextual fear memory tests. **d**

Photomicrographs showing typical examples of *c-Fos* immunostained brain sections from rats of each group at each delay. **e**, **f** *c-Fos*-positive cell density (number of cells/mm²) in the Re (**e**) or Rh (**f**) following contextual fear memory recall. ***p* < 0.01 vs 1d HC group

ReRh in remote contextual fear memory

Our results demonstrate that ReRh permanent pre-conditioning lesion specifically impaired the remote contextual fear memory. Troyner et al. (2018) also pointed to a role of the ReRh in remote memory formation, but surprisingly muscimol injection into the Re nucleus right after conditioning increased contextual freezing 21 days later and promoted memory generalization. However, the timing, i.e., right after conditioning vs pre- and post-conditioning, and the duration of ReRh silencing, i.e., transient inactivation (a few hours) vs. permanent lesion (weeks), point to major differences between the Troyner et al. study and the present's one.

In our study, ReRh permanent pre-conditioning lesion did not prevent recent contextual fear memory, but impaired remote contextual fear memory. Therefore, our data support a role of ReRh nuclei in a neuronal circuit necessary for contextual fear memory persistence, in line with our previous data regarding spatial memory (Loureiro et al. 2012). These

data thus confirm the specific implication of the ReRh in the persistence of hippocampus-dependent memories (contextual fear- and spatial memory), but not of hippocampus-independent memories (cued fear memory). Moreover, similar pattern of results was obtained after both intralaminar and anterior thalamic nuclei lesions (Marchand et al. 2014, fear memory; Lopez et al. 2009, spatial memory), suggesting a general involvement of limbic thalamic nuclei in the systems consolidation of hippocampus-dependent memories.

ReRh in retrieval of contextual fear memory

The fact that permanent ReRh lesion affected remote, but not recent memory recall performance suggests an involvement of these thalamic nuclei during either systems level consolidation processes or retrieval of the remote memory. Using a reversible DREADD chemogenetic approach, we found that the inactivation of ReRh nuclei during retrieval did not impact memory expression at any delay. Since the same

manipulation was effective in a spatial strategy-shifting task, previously shown to rely on ReRh functions (Cholvin et al. 2013), it is likely that the ReRh may not participate in fear memory retrieval processes per se, as demonstrated earlier for spatial memory with pharmacological tools (Loureiro et al. 2012; Cholvin et al. 2013). Ramanathan et al. (2018b) also showed that muscimol Re inactivation did not affect the retrieval (1 or 2-days delay) of a contextual fear memory in the original context, although they found an impairment in a novel context. Rather, their data demonstrate a participation of the ReRh nuclei to the retrieval of fear memory extinction. Indeed, the same authors (Ramanathan et al. 2018a) showed that the Re nucleus, more specifically the mPFC-to-Re projections, mediated the retrieval of recent fear memory extinction. Likewise, Jayachandran et al. (2019), who used an odor sequence task, pointed to a key role of the mPFC-to-Re pathway in sequence memory retrieval. These authors showed that chemogenetic inactivation of the mPFC-to-Re projections altered the top-down mechanisms controlling working memory.

Using brain-wide *c-Fos* mapping, Wheeler et al. (2013) extensively investigated the neuronal networks underlying remote fear memory in mice and showed that the Re nucleus appeared to be a key hub region, likely to influence overall network function during remote memory expression. Among the other regions also proposed to be such hubs are the hippocampus (mainly CA1) and the mPFC (mainly, the anterior cingulate and prelimbic sub-regions). In a follow-up study, Vetere et al. (2017) showed that chronic DREADD inactivation of the Re nucleus, starting immediately after fear conditioning, impaired remote memory performance. However, if this result confirms the crucial implication of the Re in remote fear memory, it did not establish whether its implication concerned (off-line) consolidation and/or (online) memory retrieval processes. Our chemogenetic and imaging studies—the latter showing no increase of *c-fos* expression in non-operated rats during memory retrieval whatever the post-conditioning delay—favor the first hypothesis.

ReRh in systems consolidation of hippocampus-dependent memories

The ReRh nuclei are ideally located to be a key relay between the HP and mPFC, allowing a bidirectional information flow (Varela et al. 2014) hypothesized to be necessary for systems consolidation (e.g., Squire et al. 2015; Sekeres et al. 2018). The question of the role of the ReRh nuclei in systems consolidation remains to be elucidated. Mei et al. (2018) used a crossword-like maze and showed that immediate post-acquisition, muscimol-induced ReRh inactivation did not alter recent (1 day) or remote (30 days) spatial memory. They concluded that these nuclei did not contribute to the early phase of “off-line” consolidation

processes. Noteworthy, however, is the short duration of muscimol efficiency (a few hours), which might have been insufficient to cover the entire span of the early phase of these processes.

Recent data showed that the Re nucleus controls hippocampal–prefrontal oscillatory synchrony in the delta and gamma bands. Indeed, during slow wave sleep, which is crucial for memory consolidation (Diekelmann and Born 2010; Dudai et al. 2015; Rasch and Born 2013), synchronized gamma bursts occurring within the HP and mPFC might provide a functional substrate for information transfer between these structures (Buzsáki and Wang 2012; Diekelmann and Born 2010; Dudai et al. 2015; Rasch and Born, 2013; Sirota et al. 2008). Reversible inactivation of the Re nucleus decreased the co-occurrence of gamma bursts between HP and mPFC, and fully abolished HP–mPFC gamma synchronization during slow oscillations and slow-wave sleep in the rat (Ferraris et al. 2018). Likewise, Roy et al. (2017) showed that lidocaine injection in the Re nucleus decreased coherence between mPFC and HP, specifically within the 2–5 Hz (delta) band. This low frequency oscillation is thought to provide a synchronizing signal from the mPFC to the HP via the Re nucleus. It is to note that the crucial role of the ventral midline thalamus in the hippocampal–prefrontal synchrony necessary for bidirectional communication between the dorsal HP and mPFC has also been observed during ‘online’ memory processes such as for spatial working memory-guided behavior (Hallock et al. 2016). These authors showed that Re nucleus inactivation reduced the dorsal HP–mPFC theta coherence and impaired choice accuracy in a delayed alternation task. All these data strongly suggest that consolidation impairments caused by ReRh lesion might relate to the disruption of hippocampal–prefrontal oscillatory synchrony during post-learning slow wave sleep. Moreover, Sierra et al. (2017) showed in anesthetized rats that lidocaine infusion into the Re nucleus inhibited LTP induction in the CA1–anterior cingulate cortex pathway, indicating that this nucleus might be a necessary component of the circuit underlying systems consolidation. They also confirmed that the Re nucleus plays a major role by interconnecting brain areas that control systems consolidation. Indeed, Sierra et al. (2017) showed that the Re nucleus, together with the anterior cingulate cortex, is necessary during ‘online’ reconsolidation allowing to rescue a remote contextual fear memory blocked by cortical inhibition during conditioning (Sierra et al. 2017).

Finally, we observed an increase of freezing in Sham rats after the long delay (Fig. 1e, f) as previously described (Poulos et al. 2016); this phenomenon refers to incubation (McAllister and McAllister 1967; Pickens et al. 2009). The permanent ReRh lesion could affect the incubation process accompanying memory consolidation of a contextual fear.

Conclusion

Recent evidence has pointed to a crucial role of the ventral midline thalamus in contextual fear memory specificity and persistence. A similar conclusion has been drawn from our previous study taxing spatial memory (Loureiro et al. 2012). Thus, the reorganization of hippocampo-prefrontal circuitry making a memory persistent might be supported by the ReRh nuclei regardless of the type of hippocampus-dependent memory (spatial, contextual). Altogether, the key implication of the ReRh nuclei in fear memory consolidation/reconsolidation suggests that these thalamic nuclei might be a target of choice for future therapeutic approaches to treat remote memory alterations, including traumatic memories (i.e., post-traumatic stress disorder).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the European Council Directive (2010/63/EU) and French Agriculture Ministry. All approaches have been validated by the ethical committee of the University of Strasbourg (CREMEAS—authorization #5822-2016062214582106 and #13261-2018012918394046).

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