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ORIGINAL ARTICLE

The nucleus reuniens: a key node in the neurocircuitry of stress and depression

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The hippocampus and prefrontal cortex (PFC) are connected in a reciprocal manner: whereas the hippocampus projects directly to the PFC, a polysynaptic pathway that passes through the nucleus reuniens (RE) of the thalamus relays inputs from the PFC to the hippocampus. The present study demonstrates that lesioning and/or inactivation of the RE reduces coherence in the PFC–hippocampal pathway, provokes an antidepressant-like behavioral response in the forced swim test and prevents, but does not ameliorate, anhedonia in the chronic mild stress (CMS) model of depression. Additionally, RE lesioning before CMS abrogates the well-known neuromorphological and endocrine correlates of CMS. In summary, this work highlights the importance of the reciprocal connectivity between the hippocampus and PFC in the establishment of stress-induced brain pathology and suggests a role for the RE in promoting resilience to depressive illness.

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INTRODUCTION

Disruption of the pathways linking the prefrontal cortex (PFC) and hippocampus is thought to underlie major depressive disorder.[1–4] The PFC receives a monosynaptic innervation from the ventral CA1 and subiculum of the hippocampus and there is a directionality in this communication because hippocampal activity leads the activity in the PFC.[5,6] In contrast, the reciprocal PFC output to the hippocampus is not monosynaptic but relayed via the nucleus reuniens (RE), a thalamic midline nucleus[7] (Figures 1a and b). The RE influences PFC and hippocampal activity,[8,9] presumably by modulating oscillatory patterns between these two brain structures.[10,11]

Although RE-dependent coordinated PFC–hippocampal activity was recently linked to working and spatial memory, passive avoidance learning and fear responses,[8,12–14] no information on the possible involvement of the RE on the appearance of and recovery from depressive-like symptoms is currently available.

We therefore investigated the possible involvement of the RE in depression using the chronic mild stress (CMS), a paradigm of depressive-like behavior in rodents, and the Forced Swim Test (FST), a paradigm for testing potential antidepressant interventions. Moreover, we examined the impact of RE lesioning on the synchronized activity of the PFC and hippocampus, as well as on neuromorphological and endocrine correlates of depressive-like behavior. These preclinical studies suggest that the RE occupies a central position in the neurocircuitry that underpins depression.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (3 months old, 300–350 g at the beginning of the experiment) were used. All animals were housed under controlled light/dark cycle (12:12 h, lights on at 0800 hours) and constant temperature/humidity (22 °C/30–40%). Animals had *ad libitum* access to food and water, unless dictated otherwise by specific test protocols; they were randomly selected and allocated to treatment/surgery groups, except as otherwise noted (see experiment 3). Animals were single-housed postsurgery. Behavioral tests were carried out in the light phase. All behavioral experiments and scoring, as well as neurobiological procedures were performed by raters blind to the group allocation. Procedures on animal experiments were reviewed and approved by the relevant local ethics committee and studies were carried out in accordance with European Union Directive 2010/63/*EU* on animal care and experimentation.

Surgical procedure for RE lesions. Animals were anesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine and xylazine[15] (100 and 10 mg kg^{-1}, respectively) and placed in a stereotaxic frame (David Kopf Q1 Instruments). The RE lesions were performed by injecting 0.6 µl of 100 mM *N*-methyl-D-aspartate (in 0.1 M phosphate-buffered saline (PBS), pH = 7.4; 0.1 µl min^{-1}) or vehicle (0.1 M PBS, pH = 7.4) directly into the RE (+2.3 mm AP, ± 1.7 mm ML, and − 6.2 mm DV from bregma);[16,17] the syringe was left in place for an additional 5 min to ensure adequate diffusion.[16,18–20] The RE was accessed at a mediolateral angle of 15° to avoid damage to midline brain structures and vessels, and injections were alternated between left and right angles of access to randomize possible lateralized brain damage.[21] Animals were closely monitored after surgery, returned to their home cages and allowed to recover for 1 week before further testing.

Histological verification of RE targeting. Brain sections were lightly stained with cresyl violet or hematoxylin to verify correct placement of cannulae and the extent of RE lesions. Lesion size (area) was estimated according to the presence of characteristic signs of excitotoxic reaction (loss of neurons or tissue, proliferation of microglial cells, pyknotic nuclei and accumulation of hemosiderin).[16] The area of the lesion, relative to the whole RE, was estimated using the Stereoinvestigator software (MicroBrightField), with Q2 reference to the Paxinos and Watson atlas;[17] data are shown in Supplementary Figure S2. Animals with lesion ratios < 50% were excluded

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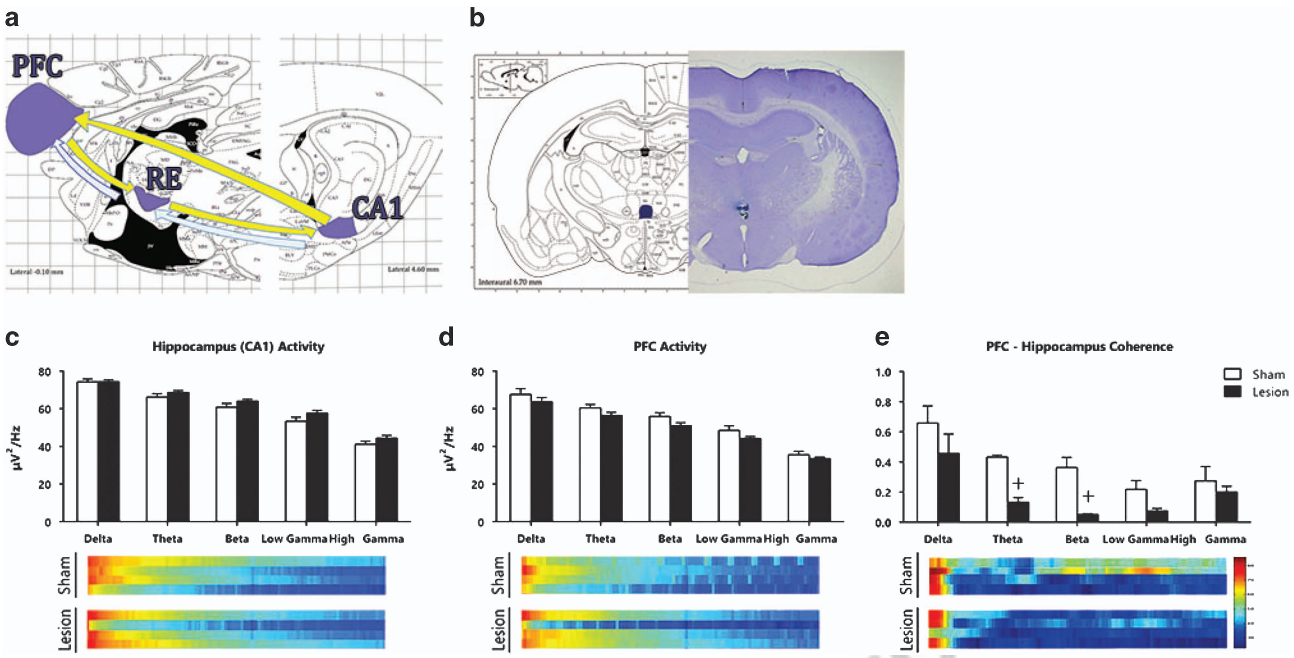

Figure 1. Nucleus reuniens (RE) lesion impacts on the function of prefrontal cortex (PFC)–hippocampus circuitry. (**a**) Schematic representation of PFC–RE–hippocampus circuitry. (**b**) An atlas reference diagram and a slice photomicrograph of the RE lesion. (**c**, **d**) Overall CA1 and PFC activity as measured by power spectrum densities was comparable in the sham and lesion groups. (**e**) PFC–hippocampus coherence was decreased in lesioned rats, in comparison to sham controls.+denotes a significant lesion effect, $P < 0.05$.

from the analysis,[16,19] and animals with a lesion covering ⩾10% of any other brain area in the vicinity of the RE were likewise excluded. Rats with sparing of the RE were excluded from the analysis (35% for the electrophysiology experiment, 11% for the FST experiments and 8% for the CMS experiments).[14,16,19]

Experiment 1: Electrophysiological studies in RE-lesioned rats

Animals ($n = 4$ per group) were anesthetized (sodium pentobarbital 60 mg kg^{-1}, i.p., supplemented every 60 min throughout the experiment) and placed in a stereotaxic frame (David Kopf Instruments); rectal temperature was maintained at 37 °C by a homoeothermic blanket (Stoelting, Dublin, Ireland). To assess RE-modulated activation and synchrony within the PFC and hippocampus pathway, platinum/iridium Q3 recording electrodes (Science Products, Germany) were placed in the prelimbic frontal cortex and a concentric bipolar tungsten/stainless-steel Q4 electrode (WPI, USA) was positioned into the ipsilateral CA1/subicular region of the ventral hippocampus (−3.3 mm AP, ±0.8 mm ML, and −4.0 mm DV from skull for the PL; +6.5 mm AP, ±5.5 mm ML, and −5.3 mm DV from skull for the hippocampus), as described previously.[2,5,22] Recorded extracellular local field potentials were amplified, filtered (0.1–300 Hz, LP511 Grass Amplifier, Astro-Med, Rodgau, Germany), acquired Q5 (Micro 1401 mkII, CED, UK) and recorded at a sampling rate of 1000 Hz on a personal computer running the Signal Software (CED). After the electrophysiological protocols, a biphasic 1 mA stimulus was delivered to both electrodes. Thereafter, rats were killed and perfused with 4% paraformaldehyde (PFA) to verify correct placement of the recording electrodes (also see below); data from animals in which electrodes were misplaced were discarded.

Experiment 2: FST in the presence of RE lesion or RE inactivation

Forced swim test. The FST, a standard test for screening the antidepressant potential of various interventions, was carried out as previously described.[23–27] Briefly, 1 week after surgical lesions or transient pharmacological inactivation of the RE, rats were placed in a cylindrical tank (60 × 19 cm^2, filled to a height of 40 cm with water at a temperature of 24 ± 1 °C) and were forced to swim for 15 min during a pretest (training) session. After 24 h, animals were subjected to a 5 min swimming session (test session).[28] Sertraline was added in this experiment, as a positive control, and sham-operated animals were given an i.p. injection of the antidepressant sertraline (10 mg kg^{-1}) or vehicle 23, 5 and 1 h before the FST test session ($n = 8$–12 per group).[29,30]

RE inactivation. To investigate whether temporary RE inactivation would have the same effects in the FST as permanent RE lesions, a different set of rats was used for RE inactivation studies during FST ($n = 8$ per group). For RE inactivation, injection needles were introduced via a stainless steel guide cannula (0.4 mm in diameter) implanted in the RE (1.0 mm above the targeted site to allow the tip of the infusion needle to protrude into the tissue).[21] Five minutes before the FST pretest or test session, 0.6 µl of tetracaine (Sigma; 2% w/v dissolved in PBS)[14] was slowly infused (3.5 min; Q6 0.2 µl min^{-1}) to prevent tissue damage using a micropump (CMA-100; CMA/Microdialysis).[14,19] To facilitate postmortem evaluation of RE inactivation, 1% w/v cresyl violet was added in the tetracaine solution used for inactivation.[14,16,19]

Open field (OF) test. This test was used to assess the impact of surgical RE lesions on locomotor activity. For this, an OF apparatus (square arena 43.2 × 43.2 cm^2) surrounded by tall Perspex walls (Med Associates, St Albans City, VT, USA) was used. Sham-operated and RE-lesioned animals were placed in the center and allowed to explore the area for 10 min. Infrared beams and the manufacturer's software were used to automatically register exploration of the arena.[23]

c-FOS immunostaining. To investigate whether the RE is activated following swim stress, c-FOS immunostaining[31] was performed on brain sections from sham-operated rats exposed to the FST. Briefly, 90 min after the last FST session, animals ($n = 5$ per group) were anesthetized and perfused with 4% PFA (in 0.1 M PBS) before careful excision of the brain, postfixation (4% PFA) and transfer to 30% sucrose (in PBS 0.1 M). After incubation in 0.3% Triton X-100/0.1 M glycine/10% fetal bovine serum, 50 µm sections were cut on a vibratome and incubated with c-FOS antibody (1:10 000; overnight, cat. no. PC05, Calbiochem, Darmstadt, Germany). Sections were then incubated in biotinylated goat anti-rabbit antibody (cat. no. E0432, Dako, Glostrup, Denmark) and Avidin/Biotin Complex (ABC solution; Vectorstain Elite, Burlingame, CA, USA). Neurons in the RE that were c-FOS-immunoreactive were counted using the StereoInvestigator software (MicroBrightField). Immunoreactive c-FOS was visualized with diaminobenzidine before light counterstaining with hematoxylin. For double-labeling experiments, c-FOS was detected by

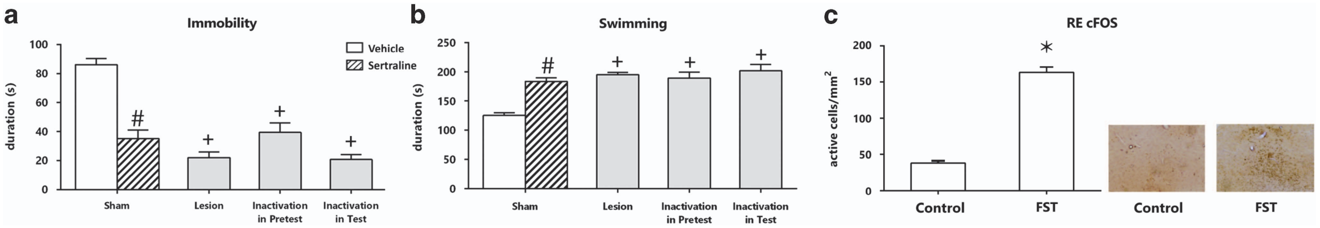

Figure 2. Nucleus reuniens (RE) lesion exhibits antidepressant effect in Forced Swim Test (FST). (**a**) Lesion of RE before FST procedure and alternatively a temporary RE inactivation either at the 'pretest' or 'test' swim session, prevented the appearance of depressive-like behavior by reducing immobility duration in the second, 'test' swim session, similar to sertraline administration. (**b**) All RE activity manipulations lengthened the active, swimming behavior, as sertraline did. (**c**) FST increased c-FOS-expressing neuron density in RE. *Denotes a significant stress effect, #a significant treatment effect and +a significant lesion effect, $P < 0.05$.

immunofluorescence. For this, antigen retrieval was achieved using the citrate buffer before overnight incubation (4 °C) of 50 µm sections
Q7 (vibratome-cut) with antisera against c-FOS (1:500; cat. no. AB1584, Millipore, USA) and calretinin (1:500; cat. No. AF5065, R&D Systems, USA) and counterstaining with 4′,6-diamidino-2-phenylindole (1 µg ml^{-1}).
Q8 Labeled cells in the RE were counted using an Olympus BX51 microscope.

Experiment 3: Effects of CMS in RE-lesioned rats

Chronic mild stress. A slightly modified version of a previously described CMS protocol[32–35] was used in RE-lesioned rats, in order to investigate the role of the RE in this model of depression (see Supplementary Table 1). Four groups of animals (control/sham-operated, $n = 15$; control/RE-lesioned, $n = 12$; CMS/sham-operated, $n = 14$; and CMS/RE-lesioned, $n = 13$) were used. During the last 3 weeks of CMS, each of these groups was subdivided ($n = 6$–8 per group); one half of each subgroup received daily i.p. injections of the antidepressant sertraline (10 mg kg^{-1} day^{-1}, as a positive control) while the other half received vehicle (0.9% saline i.p.).

Sucrose preference test (SPT). Anhedonia, a core symptom of depression, was monitored using the SPT on a weekly basis after initiation of the CMS protocol.[33,36] Animals that had been food and water deprived (18 h) were presented with two preweighed bottles, one containing a 1% sucrose solution and the other containing tap water, over a period of 1 h. Sucrose preference was calculated according to the formula: sucrose preference = (sucrose intake/(total fluid intake)) and expressed as a percentage. Following collection of sucrose preference data at week 0 (baseline), animals were assigned to the control and CMS groups, as before.[32,35] Briefly, rats were assigned to the control and CMS groups alternating from highest to lowest preference, so as the difference of means between the two groups would be the lowest possible.

OF test and FST. Twelve hours after the end of the CMS protocol, all animals were subjected to the OF test to monitor locomotor activity and 24 h later to the FST, as described above (experiment 2).

Tissue collection. Rats were anesthetized (pentobarbital) and PFA-perfused immediately after the second (test) session of the FST; just before the perfusion, a blood sample was withdrawn (under anesthesia) from the right ventricle of the heart for the eventual assay of corticosterone using a commercially available kit (ICN Biomedical, Costa Mesa, CA, USA; inte-assay coefficient of variation: 8%).[25,32,37] Adrenals were carefully dissected and weighed upon killing (Supplementary Figure S4).

Neurostructural analysis. To investigate the role of the PFC–hippocampus circuit in CMS-induced neuromorphological changes, rats exposed to CMS and their corresponding controls were perfused with saline; brains were collected and immersed in Golgi–Cox solution for 14 days before transfer to a 30% sucrose solution. Coronal vibratome sections (200 µm) were collected in 6% sucrose, dried onto gelatin-coated microscope slides, alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Linda-a-Velha, Portugal), fixed, dehydrated and mounted, as previously described.[33,38,39] Dendritic arborization, spine density and spine shape of neurons in the RE and layer II/III of the prelimbic area of the mPFC ($n = 5$ for all groups; 6 neurons per each animal) were subsequently analyzed. Briefly, for each selected neuron, all branches of the dendritic tree were reconstructed
Q9 using a motorized microscope (Axioplan 2; Carl Zeiss) and the Neurolucida software (MicroBrightField) and the dendritic length was automatically calculated. Dendritic spine density (number of spines/dendritic length) was determined in the proximal (60–120 µm) and distal (140–200 µm) parts of the apical dendritic tree. To assess changes in spine morphology, spines in the selected segments were classified into mushroom-shaped, thin, wide and ramified spines, according to Harris;[40] the proportion of spines in each category was calculated for each neuron. A Sholl analysis (index of dendritic complexity and degree of arborization) was also conducted; the number of dendritic intersections with concentric spheres positioned at radial intervals of 20 µm from the soma was assessed using NeuroExplorer software (MicroBrightField), as previously described.[33]

Experiment 4: Effects of RE lesioning before vs during exposure to CMS

Chronic mild stress. The next CMS experiment followed the same protocol as described above in experiment 3. The purpose of this experiment was to investigate whether RE lesions can prevent or reverse CMS-induced changes. A new set of animals were given a sham operation or an RE lesion, and 1 week later, they were subjected to CMS. Four weeks into the CMS, the protocol was suspended and previously sham-operated animals were given a second sham operation or received an RE lesion. Rats were allowed 3 days to recover from surgery and CMS resumed with the resulting three experimental groups: 6 rats with RE lesion before CMS, 7 rats with RE lesion during CMS, and the remaining 9 sham-operated rats. The CMS protocol continued for further 6 weeks and SPTs were performed as described above.

Dexamethasone suppression test (DST). At the end of the CMS, a DST was administered to all animals, in order to assess the glucocorticoid-negative feedback sensitivity of the hypothalamus–pituitary–adrenal (HPA) axis.[41] Briefly, all animals received an injection of either dexamethasone (100 µg kg^{-1}, i.p.) or vehicle (0.9% saline) before being subjected to a swim stress (at 24 ± 1 °C) for 15 min. Two hours later, rats were killed and blood samples were analyzed for corticosterone levels, as described above. Data are presented as the relative percentage of changes of corticosterone levels in the corresponding group pairs (dexamethasone-injected vs saline-injected).[41]

Statistical analysis

Sample sizes were determined by power analysis, based on effects sizes previously observed in previous similar experiments performed by the authors, at 80% power and type I error equal to 5%. After testing for normality and homogeneity, appropriate statistical tests were applied to the data. Repeated-measures analysis of variance was used to analyze results from the SPT. One-way, two-way and three-way analysis of variance were used, as appropriate, to evaluate other behavioral data as well as morphological, electrophysiological, hormonal and immunohistochemical results. Differences between groups were then determined by Bonferonni's *post hoc* analysis. Significance level was set at $P = 0.05$. All results are expressed as mean ± s.e.m.

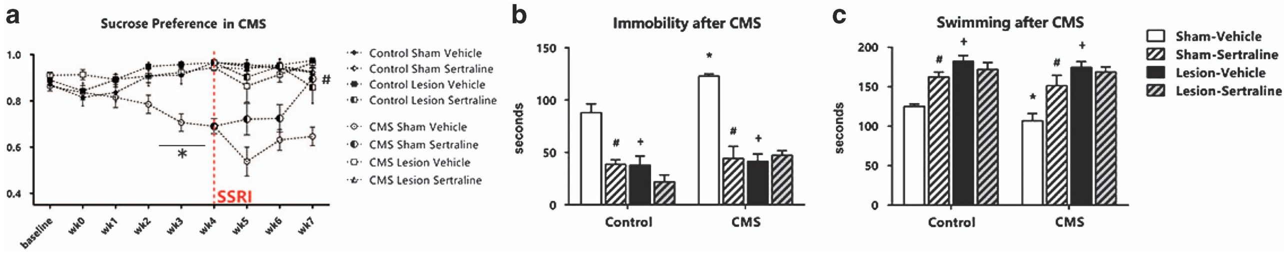

Figure 3. Nucleus reuniens (RE) is essential for depressive behavior and neuronal deficits induced by chronic stress. (**a**) RE lesion prevented chronic mild stress (CMS)-induced decreased sucrose preference. Sertraline treatment at week 4 reversed CMS-induced decreased sucrose preference. (**b**) CMS significantly increased immobility duration. Sertraline reduced immobility only in sham-operated animals and RE lesion resulted in decreased immobility only in vehicle-treated rats. (**c**) In contrast, RE lesion and sertraline increased swimming in control and CMS rats. *Denotes a significant stress effect, #a significant treatment effect and +a significant lesion effect, $P < 0.05$.

RESULTS

RE lesion impacts on the function of PFC–hippocampus circuitry and elicits antidepressant-like effects

Network dynamics in the PFC–hippocampus loop were compared between sham-operated and RE-lesioned rats by simultaneously recording neuronal activity in the medial PFC and ventral hippocampus. Power spectrum densities and coherence analyses, based on local field potentials, were used as indicators of power activity and phase coherence between the PFC and hippocampus.[5,42] As shown on Figure 1, RE lesioning did not alter overall activity in the hippocampus (Figure 1c) and PFC (Figure 1d), evidenced by monitoring power spectrum densities at several frequency bands. However, coherence between firing in the PFC and hippocampus was significantly reduced in RE-lesioned animals; specifically, as compared with their sham-operated controls, RE-lesioned animals displayed reduced theta and beta frequency bands; the same tendency was observed for gamma frequency bands (Figure 1e; lesion main effect: theta: $F_{1,6} = 82.46$, $P < 0.001$; beta: $F_{1,6} = 20.74$, $P = 0.004$ and gamma: $F_{1,6} = 5.59$, $P = 0.056$).

Using the FST, which is used widely to assess the antidepressant potential of drugs and various interventions,[43] we found that RE-lesioned animals exhibited lower immobility levels than sham-operated animals during the second FST session (Figure 2a). Interestingly, the duration of immobility observed in RE-lesioned animals was comparable to that observed in rats that received sertraline, an antidepressant drug employed in this study as positive control. Similar antidepressant effects were apparent when the RE was transiently inactivated with tetracaine either before the ‘pretest’ (first) or ‘test’ (second) FST session (Figure 2a) (inactivation main effect: $F_{3,32} = 49.50$, $P < 0.001$; *post hoc*: lesion vs sham $P < 0.001$, ‘pretest’ inactivation vs sham $P < 0.001$, ‘test’ inactivation vs sham $P < 0.001$; main effect of sertraline treatment: $F_{1,18} = 50.68$, $P < 0.001$). Rats with transient inactivation (tetracaine-induced) or permanent excitotoxic lesion (*N*-methyl-D-aspartate-induced) of the RE showed duration of swimming behavior that was greater than that observed in sham-operated rats. Moreover, swimming duration was comparable in RE-lesioned and sertraline-treated animals (Figure 2b) (inactivation main effect: $F_{3,32} = 27.63$, $P < 0.001$, *post hoc*: lesion vs sham $P < 0.001$, ‘pretest’ RE inactivation vs sham $P < 0.001$, ‘test’ RE inactivation vs sham $P = 0.002$; treatment main effect $F_{1,18} = 51.29$, $P < 0.001$). Climbing duration did not differ significantly between any of the groups (data not shown).

Complementing the above results, we observed in sham-operated rats that FST activates the RE because there was an increase in the density of c-FOS immunoreactive cells (Figure 2c; FST main effect: $F_{1,8} = 240.6$, $P < 0.001$). Interestingly, after the FST the percentage of calretinin cells that co-expressed c-FOS did not change significantly (Supplementary Figure S2; FST main effect: $F_{1,6} = 2.465$, $P = \text{ns}$).

Finally, RE lesion did not affect neither the locomotor activity, measured by ambulation in an OF arena, nor the amount of time spent in the center of the arena (lesion main effect: $F_{1,19} = 0.20$, $P = \text{NS}$ and $F_{1,19} = 0.04$, $P = \text{NS}$, respectively; Supplementary Figures 3a and b). These behavioral findings concur with previously pubished observations.[19]

Role of RE in eliciting depressive-like behavior supported by behavioral and neuromorphological measures

CMS is an acknowledged paradigm for inducing depressive-like behavior in rodents.[35] Anhedonia, which is a core symptom of depression, can be modeled in rodents using the SPT, and in agreement with numerous previous studies,[23,32] the CMS paradigm successfully decreased sucrose preference after 4 weeks. Moreover, treatment with sertraline in the following 3 weeks reversed the CMS-induced anhedonia (Figure 3a). Importantly, lesions of the RE prior to exposure to the 7-week CMS paradigm abrogated the CMS-induced anhedonia (time × CMS × lesion interaction: $F_{5,250} = 2.46$, $P = 0.034$, CMS × treatment interaction $F_{1,47} = 4.97$, $P = 0.031$, *post hoc*: sertraline–CMS vs vehicle–CMS $P = 0.025$; Figure 3a).

The duration of immobility in the FST was enhanced by CMS and decreased by sertraline in both control and CMS-exposed rats (Figure 3b). Importantly, all RE-lesioned rats (control and CMS-exposed) exhibited reduced immobility in the FST (CMS × treatment × lesion interaction: $F_{1,46} = 6.657$, $P = 0.013$, *post hoc*: lesion–vehicle–CMS vs sham–vehicle–CMS $P < 0.001$, lesion–vehicle–control vs sham–vehicle–control $P < 0.001$, sertraline–sham–CMS vs vehicle–sham–CMS $P < 0.001$, sertraline–sham–control vs vehicle–sham–control $P < 0.001$, CMS–vehicle–sham vs control–vehicle–sham $P = 0.001$). In addition, sertraline treatment and RE lesioning increased the time spent swimming in control and CMS rats (Figure 3c; treatment × lesion interaction: $F_{1,46} = 19.83$, $P < 0.001$, *post hoc*: lesion–vehicle vs sham–vehicle $P < 0.001$, sertraline–sham vs vehicle–sham $P < 0.001$). Finally, sertraline and RE lesioning reduced serum corticosterone levels (treatment and lesion main effect: $F_{1,46} = 4.64$, $P = 0.037$ and $F_{1,46} = 4.09$, $P = 0.049$; Supplementary Figure S5). Taken together, all findings show that disruption of RE function prevents the establishment of depressive-like behavior in CMS.

Consistent with the absence of CMS-induced depressive-like behavior in RE-lesioned rats, these animals did not display neurostructural changes in the PFC after CMS. Specifically, RE lesioning prevented the atrophy of dendrites of PFC neurons that follows exposure to CMS[33] (Figures 4a and b; lesion × CMS interaction: $F_{1,32} = 7.14$, $P = 0.012$, *post hoc*: lesion–CMS vs sham–CMS $P = 0.002$, CMS–sham vs control–sham $P = 0.002$, $n = 5$ per group). Similar to RE lesioning, sertraline also counteracted

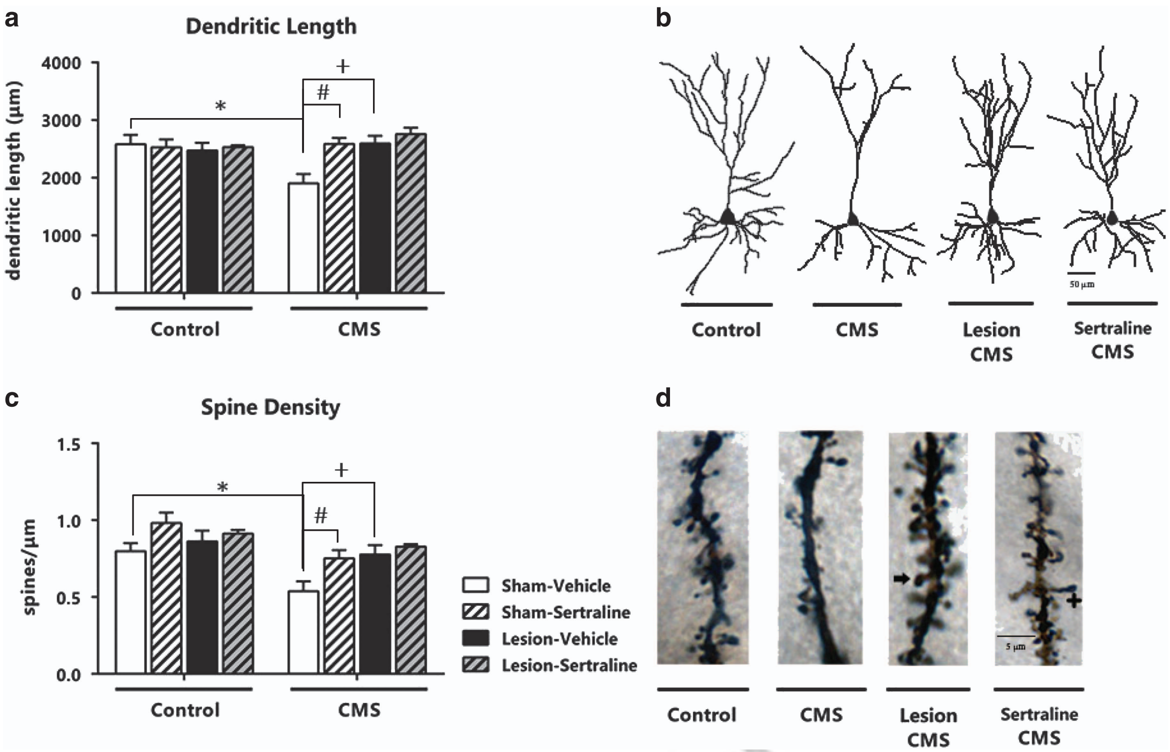

Figure 4. Chronic mild stress (CMS)-evoked dendritic deficits of prefrontal cortex (PFC) neurons were attenuated by nucleus reuniens (RE) lesion. (**a**) RE lesion prevented and sertraline reversed CMS-induced reduction of dendritic length of PFC neurons. (**b**) Depiction of three-dimensional reconstructed cortical pyramidal neurons. Scale bar: 50 μm. (**c**) RE lesion also prevented the CMS-induced spine density decrease at the apical dendrites of PFC neurons. (**d**) Representative photomicrographs of spine-bearing branches. The arrow indicates a thin spine and the plus sign a mushroom spine; *denotes a significant stress effect, #a significant treatment effect and +a significant lesion effect, $P < 0.05$. Scale bar: 5 μm.

CMS-induced dendritic atrophy in PFC neurons (Figures 4a and b; treatment × CMS interaction $F_{1,32} = 5.47$, $P = 0.026$, *post hoc*: sertraline–CMS vs vehicle–CMS $P = 0.002$, $n = 5$ per group). Sertraline itself did not affect dendritic length of RE neurons (treatment main effect: $F_{1,16} = 0.69$, $P =$ NS, $n = 5$ per group), whereas there was a tendency for CMS to increase dendritic length in RE neurons (CMS main effect $F_{1,16} = 3.94$, $P = 0.065$) (Supplementary Figure S5).

Protection against CMS-induced reductions in PFC apical dendrite spine density was another important effect that resulted from either RE lesioning or sertraline treatment (lesion × CMS interaction: $F_{1,32} = 4.82$, $P = 0.035$, *post hoc*: lesion–CMS vs sham–CMS $P = 0.006$, sham–CMS vs sham–control $P < 0.001$, treatment × CMS interaction: $F_{1,32} = 5.36$, $P = 0.027$, *post hoc*: sertraline–CMS vs vehicle–CMS $P = 0.001$, $n = 5$ per group; Figures 4c and d). As shown in Figure 5, in sham-operated animals CMS slightly reduced, whereas sertraline significantly increased the percentage of mushroom spines in the proximal part of apical dendrites in the PFC (CMS main effect $F_{1,16} = 4.38$, $P = 0.053$; treatment main effect $F_{1,16} = 4.72$, $P = 0.045$; Figure 5a). These effects were not evident in RE-lesioned animals (CMS main effect $F_{1,16} = 0.56$, $P =$ NS; treatment main effect $F_{1,16} = 3.04$, $P =$ NS; Figure 5a). Importantly, whereas CMS decreased the relative number of mushroom spines, RE lesions prevented this effect (*post hoc*: sham–CMS vs sham–control $P = 0.031$; lesion–CMS vs lesion–control $P =$ NS). Sertraline increased the percentage of mushroom spines at the distal segments of apical dendrites in all, but the CMS RE-lesioned animals (*post hoc* control: sham–sertraline vs sham–vehicle $P = 0.004$; lesion–sertraline vs lesion–vehicle $P = 0.024$; CMS: sham–sertraline vs sham–vehicle $P = 0.037$).

Results from Sholl analyses showed that dendritic arborization of the apical dendrites of PFC neurons was similarly increased by both RE lesioning and sertraline treatment in comparison to sham-operated and vehicle-treated rats, respectively (Figure 5e; $F_{2.47,78.90} = 3.36$, $P = 0.031$ and $F_{2.47,78.90} = 3.00$, $P = 0.045$, lesion and treatment main effect, respectively). Thus, similar to sertraline treatment, RE lesions spare PFC neurons from CMS-induced reductions in the dendritic complexity of PFC neurons.

RE lesions prevent, but do not mitigate, CMS effects

Having demonstrated that the manifestation of depressive-like behavior after CMS depends on an intact RE, we next asked whether the CMS-induced depressive-like behavior and HPA axis dysregulation could be reversed or ameliorated by introducing RE lesions not before but during CMS. In this second CMS experiment, we successfully repeated our previous CMS finding, as animals with an RE lesion before CMS exposure did not exhibit anhedonia and had higher sucrose preference compared with sham-operated animals (lesion main effect: $F_{1,20} = 5.148$, $P = 0.034$). Interestingly, animals that received an RE lesion during CMS were not different from sham-operated animals, thus exhibiting anhedonia that did not appear if RE lesion was performed before CMS (lesion main effect: $F_{2,19} = 4.676$, $P = 0.022$, *post hoc*: sham vs pre-CMS lesion $P = 0.032$, sham vs during CMS lesion $P = 1.0$; Supplementary Figure S6a). Moreover, CMS has been shown to elicit HPA axis dysregulation[44,45] similar to the one often seen in depressed patients.[46] Therefore, we employed the DST to monitor the expected disruption of the negative feedback of the HPA axis while under CMS.[47] In accordance with the behavioral resilience to CMS, animals with an RE lesion before CMS displayed a suppressed corticosterone response following dexamethasone despite CMS. Instead, sham-operated rats and rats that received

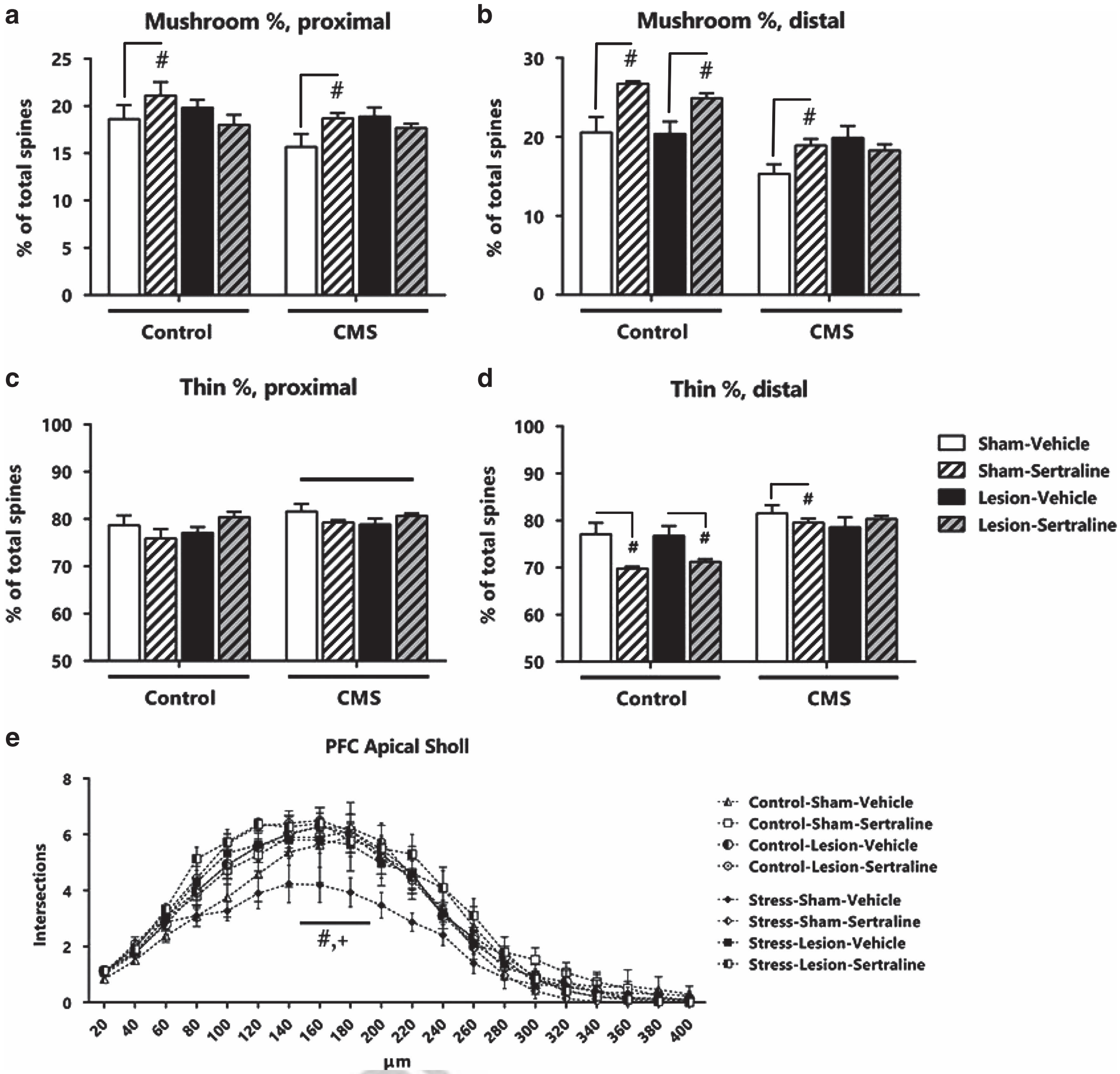

Figure 5. Impact of nucleus reuniens (RE) lesion on spine morphology and dendritic arborization. (**a**) Chronic mild stress (CMS) marginally reduced and sertraline clearly increased mushroom spine percentage in the proximal part of the apical dendrites in sham- but not in lesion-operated rats. (**b**) In the distal portion, CMS sham-operated but not lesioned rats had decreased mushroom spine percentage. Sertraline increased the mushroom percentage in all cases except CMS lesioned animals. (**c**) In the proximal portion of the apical dendrite in PFC pyramidal neurons, CMS uniformly elevated thin spine percentage. (**d**) In the distal part, sertraline treatment reduced and CMS increased thin spine percentage in sham-operated rats while in RE-lesioned rats sertraline decreased thin spine percentage only in controls. (**e**) RE lesion and sertraline increased dendritic arborization. *Denotes a stress effect, #a treatment effect and +a lesion effect.

an RE lesion during CMS displayed the depressive-like non-suppression in the DST (DST main effect: $F_{2,6} = 23.529$, $P = 0.001$, *post hoc*: lesion before CMS vs sham: $P = 0.003$, lesion before CMS vs lesion during CMS: $P = 0.003$; Supplementary Figure S6b). Taken together, findings from this experiment suggest that the RE is essentially involved in the establishment phase of depressive symptomatology rather than in processes recruited for recovering from depression.

DISCUSSION

The present experimental study provides novel evidence for the intermediary, but pivotal, role of the RE in synchronizing communication between the PFC and hippocampus. In this regard, the data presented here support and extend previous suggestions that the RE thalamic nucleus forms an integral part of the PFC–hippocampal circuitry.[8,9] Specifically, we show that the RE is essential for maintaining phase coherence between the PFC and the hippocampus. We also report that the RE has a crucial role in the manifestation of a depressive-like state and related behavioral, neuromorphological and endocrine effects. Although previous authors suggested RE involvement in the processing of emotional and cognitive information,[11,48] our observations are important because they pinpoint a neuroanatomical network that may be targeted to increase resilience against mood disorders, such as major depression.

The suggestion that the RE is implicated in the PFC/hippocampus-dependent behavioral response is supported by our finding that the FST paradigm, which enhances corticosterone levels,[25,49] leads to a significant RE activation. This is in line with a previous finding that a short exposure to an acute stressor activates the RE[50] and suggests a role of the RE in the stress response. Also relevant is to notice that an earlier report showed that antidepressant-like effects are elicited by lesions of the ventral PFC;[51] in our study, we triggered an antidepressant response, namely, reduced immobility and increased swimming duration in the FST and abrogation of anhedonia in the CMS,[52] not by lesioning the PFC but instead by lesioning a thalamic nucleus at the interplay between PFC and hippocampus.

Notably, the RE lesion and antidepressant treatment triggered behavioral responses of comparable effect size. However, it is important to note that RE inactivation at any of the two FST sessions (pretest and test) resulted in the same antidepressant-like behavioral response. Interestingly, the anhedonia during CMS, the depressive-like behavior in the FST after CMS and the disruption of the HPA axis could only be prevented when the RE lesions preceded CMS. In contrast, lesions of the RE midway through the CMS protocol failed to reverse the behavioral and endocrine anomalies induced by CMS. These observations point not only to the critical role of the RE in the stress response and its detrimental effects but may also relate to differences between the two models (FST, CMS).[52] Although CMS is known for its face and construct validity, more closely modeling the human condition, the FST excels for its predictive validity of potential antidepressant manipulations, either before or in between the two FST sessions.

Importantly, along with the behavioral resilience, RE lesions also prevented in the PFC the appearance of CMS-induced deficits in neuroplasticity (for example, dendritic atrophy and spine loss), which have been associated with depressive-like behavior.[33,53] It should be noted here that the RE predominantly projects to superficial layers of the PFC,[54] which are the most affected by CMS.[53,55–57] It is thus suggested that, in rats with an intact RE, the depressive-like morphological (plasticity) changes observed after CMS in PFC neurons may be a result of the CMS-induced change on the PFC–hippocampus crosstalk. Importantly, antidepressant (sertraline) treatment and RE lesion resulted in a similar morphological alteration of plasticity indices, such as spine density and dendritic arborization. This suggests that a PFC–hippocampus decoupling and an antidepressant treatment may partially share a common underlying mechanism of action, however, with a significant difference: PFC–hippocampus decoupling may prevent the establishment of depressive-like symptoms, whereas antidepressant pharmacotherapy may prevent and restore depressive-like symptoms in animal models of depression. Moreover, our findings on the DST are consistent with the experimental and clinical data, which demonstrate that often an altered HPA axis negative feedback associates with the appearance of depressive-like symptomatology.[58] Taken together, these findings show that the prevention of depressive-like behavior by RE lesion extends not only to the behavioral response but also to neuroendocrine and brain neuroplasticity findings that are highly related to the pathophysiology of depression.

In light of the recently emerging view that chronic stress shifts the overall brain connectome, it is relevant to examine the involvement of RE on the suggested switch between circuitries along the transition from acute stress condition to chronic stress brain construct. For this purpose, it is relevant to explore the contribution of different RE neuronal populations to this effect. Previously, it was demonstrated that calretinin-stained neurons project in the hippocampal CA1 region.[59] In this study, calretinin staining showed a high degree of co-localization with c-FOS-activated cells, thus highlighting the involvement of RE glutamate interneurons at the PFC–hippocampus communication. Lesioning of these interneurons produced the resilience to depression presented here. However, a limitation of this study is that, in contrast to humans, rodents may be virtually devoid of GABA interneurons in the RE relay nucleus.[59,60] Thus it is not yet clear whether disrupting both GABA and glutamate RE interneurons or specifically the later subpopulation would replicate our findings in humans. Finally, given the observed RE activation during FST in sham-operated animals, an optogenetic-based approach for activating RE during FST and /or CMS would also provide additional insight.

In conclusion, the present work pinpoints the RE as an important relay station in PFC–hippocampus communication and demonstrates that the refinement of cortical information flow by this specific thalamic nucleus is critical for mood regulation as well as the establishment of depressive-like pathology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

VK contributed to the design of the study, performed all experimental procedures, statistical analyses and compiled the first draft. NK contributed to the design of the study, the analysis and interpretation of results and, with AV, participated in some of the experiments; JFO and VMS helped with the electrophysiological analyses. IS and HL-A contributed to the histochemical analyses. IS and OFXA helped with the studies involving stress and data interpretation. ZP-D, KA and NS participated in study design and interpretation of results and provided significant insights. CD supervised and contributed to all parts of this project. All authors contributed to the writing of the manuscript and approved the final manuscript.

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