Brain Stimulation 12 (2019) 87–95

Contents lists available at ScienceDirect

Brain Stimulation

journal homepage: http://www.journals.elsevier.com/brain-stimulation

Prefrontal cortex rTMS reverses behavioral impairments and differentially activates c-Fos in a mouse model of post-traumatic stress disorder

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ARTICLE INFO

Article history:
Received 19 January 2018
Received in revised form
28 August 2018
Accepted 6 September 2018
Available online 8 September 2018

Keywords:
rTMS
Post-traumatic stress disorder
vmPFC
Infralimbic cortex
Basolateral amygdala
CA1

ABSTRACT

Background: Post-traumatic stress disorder (PTSD) is a severe mental illness correlated with alterations in fear extinction neurocircuits that involve prefrontal, amygdala and hippocampal structures. Current treatments indirectly restore prefrontal control of fear responses, but still cannot achieve full remission in all patients.
Objective/hypothesis: Repetitive TMS (rTMS) can directly and chronically act on subparts of the prefrontal cortex (PFC) as a potential alternative treatment. However, preclinical studies are needed to further the comprehension of its mechanisms and thus enhance its efficacy.
Methods: A 40-mm coil is used on a stereotaxic frame to apply 12-Hz high-intensity rTMS of the ventromedial PFC (vmPFC) in a foot-shock mouse model of PTSD. Chronic rTMS treatment was applied 7 days after the shocks every day up to day 12 (5 sessions, 3750 pulses).
Results: One session of rTMS (750 pulses) was able to precisely evoke immediate c-Fos activity in an area of the vmPFC (0.5 mm^2) in preliminary control mice. When used in the foot-shock model, chronic rTMS treatment ($n = 19$) counteracted short-term episodic memory deficits at day 18, and enhanced extinction dynamics when reexposed to the shocking chamber at day 22. Associated c-Fos activity was found increased in the rodent's vmPFC (infralimbic cortex), the basolateral amygdala and the ventral CA1 (hippocampal output).
Conclusions: This study is the first to use prefrontal cortex rTMS in a mouse model of PTSD. Chronic rTMS of the vmPFC reversed stress-induced behavioral impairments and acted on distributed networks of fear extinction up to 10 days after treatment.

Introduction

Post-traumatic stress disorder (PTSD) is a chronic psychiatric condition that occurs after sustaining or witnessing a trauma. Individuals with PTSD have been shown to exhibit reexperiencing symptoms, impaired memory, attentional deficits and smaller hippocampal volumes than control populations [1]. The disorder has been extensively described through the framework of fear conditioning and extinction [2], which mainly involves neural interactions between prefrontal, amygdala and hippocampal structures [3,4]. Rodent studies have confirmed the pivotal role of the prefrontal cortex (PFC) in the pathophysiology of PTSD [5], specifically the involvement of its ventromedial subpart in fear extinction [6,7], as well as the contribution of its efferences to the amygdala and its afferences from the hippocampal CA1 subarea [8].

First-line care includes trauma-focused exposure therapy, with strong treatment responses based on fear extinction mechanisms [9]. It is widely accepted that many PTSD patients are treated with antidepressant compounds (selective serotonin reuptake inhibitors, SSRIs), but only one third of patients achieved remission [10,11]. Despite positive outcomes, no current therapy addresses the neurobiological correlates of fear extinction. Repetitive transcranial magnetic stimulation (rTMS) can act non-invasively on

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https://doi.org/10.1016/j.brs.2018.09.003
1935-861X/

cortical structures [12]. Targeted electromagnetic fields are described to depress or potentiate long-term synaptic communication near a 5-Hz watershed [13–15]. Their use is thus of prime interest as an evidence-based treatment for neuropsychiatric conditions [16,17]. Through repeated interference with the dorsolateral PFC, rTMS can counteract dysfunctions of distributed networks associated to PTSD [18]. Raij et al. (2017) recently paired high-frequency rTMS to the ventromedial PFC with a conditioned cue to enhance fear extinction in humans [19]. However, and as a technique mostly built upon empirical data, rTMS patterns often vary between protocols, which can lead to different therapeutic outcomes [20,21]. Preclinical research could further the comprehension of key mechanisms at play between rTMS and pathological correlates of PTSD.

Contextual fear conditioning models have been used successfully to evoke PTSD-like symptoms in mice [22–24], with behavioral shifts that can be reliably assessed up to one month afterward (e.g., memory impairment, hallmark freezing behavior). Nevertheless, rTMS has only been scarcely evaluated in mice due to a poor adaptation of available magnetic transducers to the smaller-sized rodent brain [25,26] and an intrinsic over-heating of down-scaled coils that aim to fit those dimensions while maintaining high intensity parameters [27,28]. Despite focality biases, the use of human-purposed coils on rats have, to some extent, yielded significant effects on synaptic potentiation [29], acted on c-Fos neuronal reactivity patterns [30], and lately, reversed cingulate cortex signaling abnormalities in a PTSD model after application of a high-frequency pattern [31]. Some studies employed pulsed fields in the milli-Tesla range to obtain higher spatial resolution [32,33]. However, these transducers lack the high-rate, high-intensity trade-off relative to bigger coils [34,35].

Considering this current background, the aim of the study was to 1) create an rTMS protocol that can focally act upon the vmPFC in mice, and to 2) test its potential efficacy on key behaviors and associated neuronal activity in a mouse model of PTSD. To that purpose, an actively-cooled transducer able to generate focal, dense and high-rated patterns [36], was used through stereotaxic framing to activate the vmPFC in Swiss mice, verified by c-Fos functional mapping of the cortical target and its surroundings. The rTMS protocol was then extended and administered chronically (5 days) to a second cohort of Swiss mice that underwent foot-shock contextual fear conditioning. The effects of the focal, high-intensity/frequency rTMS treatment, was assessed on episodic-like memory (object recognition task) and extinction dynamics (reexposure to the conditioning context). Finally, the neuronal activity associated to the reexposure event was characterized with c-Fos immunolabelling of the PFC, amygdala and hippocampal output (CA1), key structures in fear extinction.

Material & methods

Animals and study design

One hundred and fifty-five male Swiss mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France), aged 9 weeks (36 ± 4.5 g) at the beginning of the experiments. The mice were group-housed by four in Makrolon Type III cages at room temperature 22 ± 1 °C and had free access to tap water and food pellets. Experiments were conducted during the dark phase of a 12:12 light-dark cycle. All procedures were compliant with Directive 2010/63/EU guidelines on animal ethics (referral 04808, approved by the ethical committee CEEvdl).

Fifteen mice were allocated to a first procedure (Experiment 1), designed to set up a high-intensity/frequency rTMS protocol able to focus the PFC at bregma +2 mm [37] and act on its activity distribution immediately. One hundred and forty mice then underwent a 22-day long core experiment (Experiment 2, Fig. 1A), designed to evaluate the effects of the rTMS protocol as a chronic treatment in a foot-shock conditioning model of PTSD (Non-stressed/"NS" n = 69, Stressed/"PTSD" n = 71). Chronic rTMS was compared to the classic SSRI treatment fluoxetine.

Magnetic stimulation setup

The TMS bundle was acquired from the manufacturer MagVenture (A/S, Denmark). The transducer (Cool-40 Rat Coil) used in all procedures was designed and specified by Parthoens et al. [36]. The coil is circular (40 mm) and folded 20° from the orthogonal plane to enhance spatial resolution. A MagPro R30 generator provided a mean output (MO) that ranges from 2 to 190 A/µsec. In a rat model, described to react similarly to a mouse model in this context [25], the electrical field induced under the brain surface was previously estimated at 100 V/m for the maximal MO [36], a strength comparable to parameters employed in humans [38]. Electrical current flowed clockwise into the coil, hence counter-clockwise on the brain surface. All TMS procedures were done under 1.8 L per minute gaseous isoflurane anesthesia (1%, halogenated ether, Aerrane, Baxter SAS). To provide a reproducible mouse-coil interface, the mouse was placed on its ventral side in a stereotaxic apparatus (SM-6M-HT, Narishige). The head was non-invasively bolted to the stereotaxic frame with auxiliary ear bars (EB-5N, Narishige).

Experiment 1: one-session rTMS to the PFC

Stereotaxic interface. To provide reproducible positioning, the TMS coil was moved on the stereotaxic frame by a custom-made

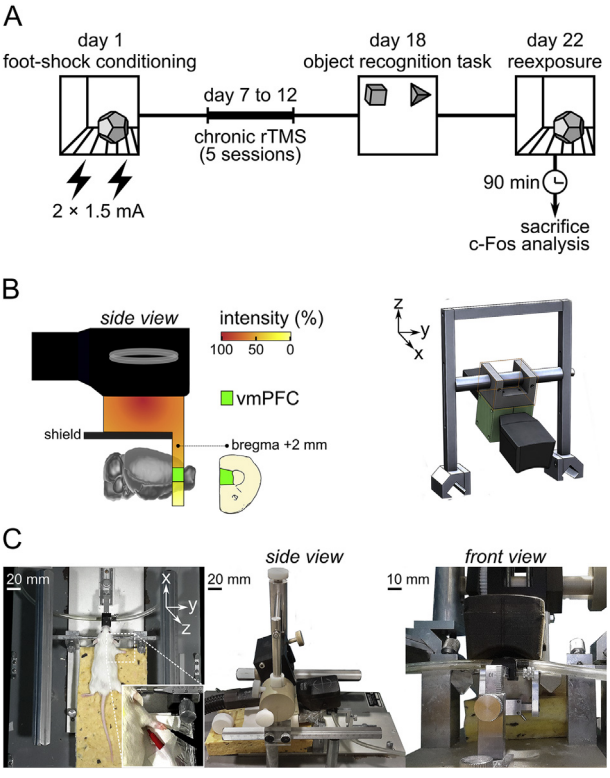

Fig. 1. Study design. (A) Timeline of Experiment 2. (B) Left: unscaled view of the transducer and the shielded brain. Right: stereotaxic manipulator. (C) Photographs of the stereotaxic frame (left inset: MEP electrodes positions) and the stereotaxic manipulator holding the transducer (middle and right).

manipulator (Fig. 1B), machined from SLS-printed polyamide and amagnetic hardened aluminum alloy (AU4G). The manipulator generated 3 linear degrees of freedom (x, y, z) and a rotation of the xy plane. Movements of the transducer were based on the center of the coil and operated with 1-mm stepped threading. The fourth degree of freedom (rotation of xy) was designed to align the coil tangentially to the varying rostro-caudal curvature of the cranium. To further spatial resolution, non-targeted parts of the nervous system were shielded with a 0.5 mm thick nickel/iron alloy sheet (MagnoShield FLEX+, Aaronia) [39]. Because circular coils generate torus-shaped electromagnetic fields [40], the intensity distribution was taken into account for the stereotaxic interface. To produce universal positioning vectors, the relative position of the primary motor cortex (M1) was thus mapped according to anatomical landmarks. Stimulations were found most effective when using the bottom periphery and the left periphery of the coil, inducing respectively latero-medial and antero-posterior currents on the cortical surface (Supplementary A).

Motor thresholds. The intensity threshold at which the electromagnetic field elicits neuronal depolarization was determined by targeting the M1 forepaw representation of eight mice with antero-posterior induced currents (left periphery of the coil). To that purpose, a signal recording unit (MEP Monitor, MagVenture A/S) was added to the installation to fetch motor potentials (MEPs) evoked by single-pulse TMS. The MEP system was earthed and linked to a subdermal active electrode above the brachioradialis group and a subdermal reference electrode between the third and fourth carpo-metacarpal joints (30-gauge; Fig. 1C) [41,42]. A foil shield was used to protect the brainstem, the spine and the implanted limb to avoid artifacts. From a stepwise procedure akin to Mills-Nithi's algorithm [43], a sigmoidal dose-response curve was produced and the motor threshold (MT) was found at 70 A/μsec (39% MO). The first intensity step to reach plateau of the sigmoidal fit was 115% MT (80.4 A/μsec, 45% MO; Supplementary B).

Stimulation parameters. Repetitive protocols in humans have used stimulation intensities either below (80–90%) or above the MT (105–120%) to produce significant outcomes in neuropsychiatric paradigms [44,45]. Subthreshold parameters are described to behave alike suprathreshold's, although the former might act preferentially on interneurons [46] or impart weaker effects on-site [47]. Accounting for 1) strength-decay as a function of coil-to-cortex distance [48] and 2) the dose-response relationship of motor responses, the stimulation intensity chosen for the rTMS session was defined at 115% MT. Because stimulation parameters determined in one structure (i.e., M1) cannot be readily translated to another structure [49], the effects of a high-intensity/frequency rTMS pattern on the PFC needed to be assessed before engaging in treatment paradigms. The transducer was centered on the interhemispheric line and aimed at bregma +2 mm, a brain cluster that contains the rostral part of the cingulate cortex (Cg), the prelimbic cortex (PrL) and the infralimbic cortex (IL), analog of the vmPFC in rodents [50,51]. A foil shield, starting at bregma +1 mm, was used to protect parts of the brain that were posterior to the target. In contrast to MT determination, stimulation of the PFC was latero-medial to promote bilateral effects, elicited via the bottom periphery of the coil. Using biphasic pulses, which require less energy than monophasic at higher pulse repetition frequencies (PRF), the most intense, cumulative pattern obtainable with the present setup before over-heating was a rate of 750 magnetic pulses, organized in 30 trains of 25 pulses delivered at PRF 12 Hz. The train repetition frequency (TRF) was 0.07 Hz (inter-train interval: 14 s) for a total duration of 7 min and 48 s (one session). Four mice received one session of stimulation ("One-session rTMS"), while three mice received one sham session ("One-session Sham"), that is, solely anesthesia with a hovering loudspeaker reproducing the loud discharge noise of the coil. The mice were sacrificed 90 min post-stimulation to assess c-Fos reactivity patterns, an early-activated gene expressed protein that peaks 1–2 h after inducement [52].

c-Fos labelling. To prepare neural tissue for immunohistochemistry (IHC), mice were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate buffer 0.1 M (PB) for 10 min. The brains were then harvested, post-fixed in PFA 4% overnight, cryoprotected in sucrose 20% for 72 h and snap-frozen with dry-ice-cooled 2-methylbutane. Forty-micrometer coronal slices were produced from a −20 °C refrigerated microtome (cryostat, Leica CM 3050 S) for free-floating IHC. After endogenous peroxydase blockade (20 min, 50% EtOH, 1% H_2O_2), sections were processed with primary antibodies directed against c-Fos (1:1000, SC-52-G goat polyclonal IgG, Santa Cruz Biotechnologies) in PB 0.1 M, 2% Normal Donkey Serum and 0.1% Triton for 48 h at 4 °C. Then a secondary incubation (1:500, Biotin-SP-conjugated AffiniPure donkey anti-goat IgG, Jackson ImmunoResearch) was performed 2 h at room temperature. Finally, a standard protocol was used with 1-h 1%-avidin/1%-biotin complex (Vectastain Elite ABC kit) and 3,3′-di-amino-benzidine revelation (SIGMAFAST™ DAB tablets, Sigma-Aldrich) for 3 min.

Spatial distribution analysis of c-Fos reactive neurons. The immunolabelled sections were observed under a Zeiss Z.2 Imager microscope in transmitted-light mode (2.7 V HAL, 800 μsec exposure). Micrographs (magnificence ×10) were exported to the processor ImageJ [53] in grayscale 8-bit format, stitched for surface reconstruction [54], and converted to a binary mask at 60% of background's mean gray value. The spatial spread of rTMS-evoked activity was assessed with a technique derived from Tufail et al. [55]: the c-Fos + surface distributions were mapped on 6.25 mm^2 topographical grids (100 tiles of 250 × 250 μm) where counts were expressed as cellular densities (c-Fos + cells/0.0625 mm^2). Three ubiquitous sections distributed between both hemispheres (bregma +2.6 mm, target: bregma +2 mm and bregma +1.2 mm) were picked to estimate One-session rTMS spatial spread on the PFC target (Fig. 2A).

Experiment 2: chronic rTMS treatment

Foot-shock conditioning. At day 1 of the core experiment, mice underwent the acute stress of foot-shock conditioning. The protocol was described to produce PTSD-like symptoms (sleep disturbance, avoidance) in Swiss mice up to 28 days afterward [22]. Two foot-shocks (2-sec long) were administered at a 6-sec interval through a grid floor after free exploration (190 s) of the context, enriched with a gray-colored polyhedron. The mice returned to their home cage 60 s after the shocks.

Treatments. From the second day on, pharmacological treatment (Flx) or vehicle (Veh) was given through drinking water (fluoxetine, 15 mg/kg). To ensure a similar intake between mice, drinking inputs and mice were weighted two times a week. From day 7–12, five rTMS sessions or sham sessions were applied 24 h apart. The rTMS sessions were done under isoflurane anesthesia (1%), while sham sessions consisted of anesthesia only with a loudspeaker reproducing the sound of the coil. The five treatment sessions thus cumulate 3750 magnetic pulses for each mouse. Each experimental group was composed of 18 ± 1 mice (NS/PTSD Veh, Flx, Sham, rTMS).

Object recognition task (ORT). At day 17 and 18, mice underwent the ORT. The protocol employed was based on Leger et al. [56] guidelines and previously described procedures in the Swiss strain [57]. Mice were habituated to an empty, dimly lit (20 ± 2 lux) square open-field for 7 min on day 17, then familiarized with two identic objects (either two gray cubes or pyramids) in the same

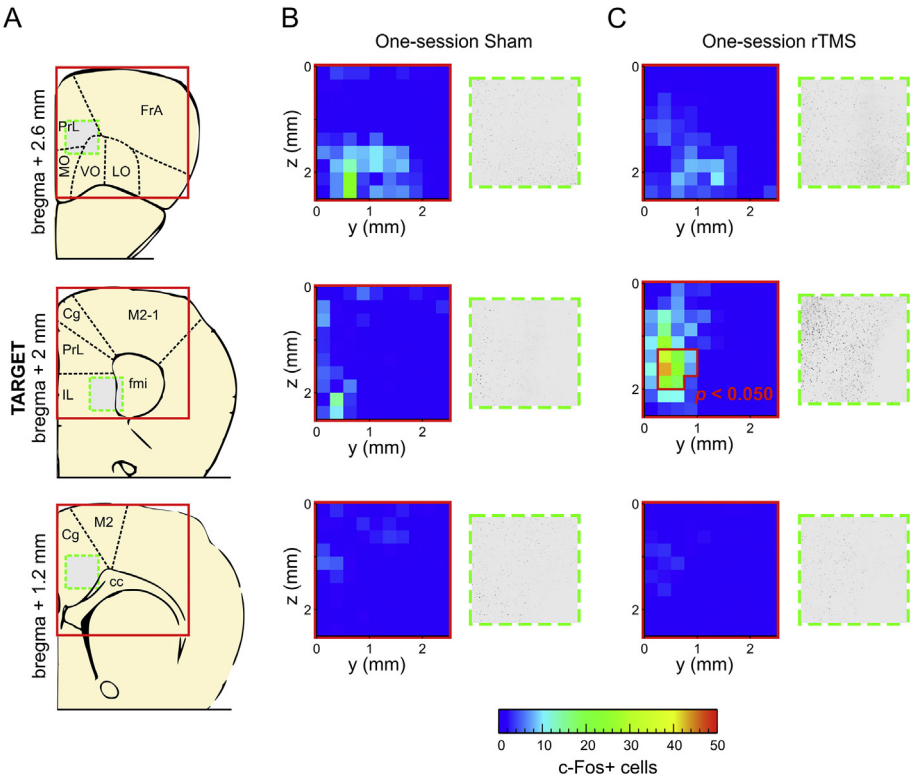

Fig. 2. Averaged c-Fos topographical maps. (A) Coronal sections picked for assessing One-session rTMS focality (target: bregma +2 mm). Appended red squares illustrate the position of the counting grids; dashed green squares illustrate a representative subfield. (B) Topographical maps showing the averaged c-Fos scores of each group and (C) selected micrographs of representative subfields. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

conditions on day 18. The mice were removed from the apparatus when 15 s of exploration were cumulated on the identical objects. Following a 1 h intersession interval, the memory trace was assessed during the retrieval session: one familiar object was swapped (e.g., cube into pyramid) and remembering the two objects was assumed when the novel object was preferentially explored [56]. The retrieval session lasted up to 5 min; to ensure similar exploration times between individuals, the retrieval session was stopped when 15 s of exploration were cumulated.

Reexposure. At day 22, mice were placed in the shocking chamber and thus re-exposed to the conditioned context. For 300 s, behavioral items relative to freezing, the hallmark behavior of fear expression, were recorded. The latency to freeze and the total duration of freezing were analyzed. Ninety minutes post-reexposure, the mice were sacrificed to observe challenged c-Fos neuronal expression.

c-Fos analysis of fear neurocircuits. Five to six mice were allocated to each experimental group for c-Fos analysis. To analyze distributed c-Fos activity patterns associated to reexposure, ubiquitous sections were picked for PrL (bregma +2.6 to +1.5 mm), IL (bregma +2 to +1.5 mm), Cg (bregma +1.6 to +1 mm), basolateral amygdala (BLA) and central amygdala (bregma −0.8 ± 0.2 mm), dorsal CA1 (bregma −1.4 to −2.8 mm) and ventral CA1 (bregma −3.5 ± 0.2 mm). Both hemispheres were evenly distributed between the analyzed sections. Counts were obtained with ImageJ (procedure detailed in Experiment 1) and expressed as normalized cellular densities (c-Fos + cells/mm^2).

Data analysis

Results are displayed as mean ± SEM on bar graphs. In Experiment 1, topographical maps display the averaged c-Fos densities for the two groups (One-session rTMS and One-session Sham). The comparison of each 250 × 250 μm tiles between groups was performed with a two-sample *t*-test. Exploration time of the novel object in the ORT (Experiment 2) was analyzed with a univariate *t*-test [56], computed against the chance level 7.5 s (50:50 familiar-novel exploration over 15 s). Remaining data was processed with a one-way ANOVA then a *post hoc* Tukey for multiple comparisons between groups: relevant comparisons were considered NS *vs* PTSD (e.g. NS Veh *vs* PTSD Veh), treated *vs* untreated (e.g. PTSD Sham *vs* PTSD rTMS) and Flx *vs* rTMS (e.g. PTSD Flx *vs* PTSD rTMS). The effect sizes of treatments were further analyzed with Cohen's *d* and given along statistical results when relevant.

Results

One-session rTMS acted focally on c-Fos reactive functional maps

Spatial spread analysis of One-session rTMS-evoked c-Fos activity yielded no significant results when averaged on full topographical grids and thus for the three measured loci, although scores at target site (bregma +2 mm) were qualitatively increased twofold compared to One-session Sham mice (Sham = 2.2 ± 1.0 *vs* rTMS = 5.2 ± 2.3 c-Fos + cells/0.0625 mm^2; t = 2.30, p = 0.07). Proportionate values were observed for topographical grids at distance from target: bregma +2.6 mm (Sham = 3.8 ± 2.3 *vs* rTMS = 3.2 ± 1.3 c-Fos + cells/0.0625 mm^2; t = 0.73, p = 0.50) and bregma +1.2 mm (Sham = 1.3 ± 0.4 vs rTMS = 0.9 ± 0.4 c-Fos + cells/0.0625 mm^2; t = −1.16, p = 0.30). Analysis of each tiles (0.0625 mm^2) on target site revealed that only a specific subfield of 0.5 mm^2 was significantly increased (Fig. 2B–C): One-session rTMS mice displayed a higher c-Fos score for eight tiles that included overlapping units of ventral PrL (3 tiles) and IL cortices (5 tiles).

Ventral PrL tiles had a mean fold value to One-session Sham of 10.5 ± 2.2, while IL tiles had a mean fold value of 11.8 ± 4.1. Tile analysis was non-significant ($p > 0.3$) in the cingulate cortex (Cg) or in structures flaring from midline (motor cortices M1 and M2), although localized closer to the brain surface.

Chronic rTMS reversed short-term memory impairments and enhanced fear extinction

ORT. Non-stressed Veh, Sham, and rTMS mice displayed memory integrity in the retrieval session of the ORT when compared against the chance level ($t = 4.15$, $p = 0.0004$; $t = 2.94$, $p = 0.005$ and $t = 4.75$, $p = 0.0001$, respectively). Non-stressed Flx mice, however, spent an equal time between the known and the novel object ($t = -0.63$, $p = 0.729$). PTSD Veh, Flx and Sham mice did not explore the novel object above the chance level ($t = -1.19$, $p = 0.87$; $t = -0.46$, $p = 0.67$ and $t = -2.14$, $p = 0.98$, respectively). PTSD rTMS mice, on the one hand, explored the novel object above the chance level ($t = 5.40$, $p < 0.0001$), and on the other hand, explored the novel object significantly more than the PTSD Sham group and the PTSD Flx group ($F(7, 140) = 8.78$, $p < 0.0001$; PTSD Sham *vs* PTSD rTMS, $p < 0.0001$; PTSD Flx *vs* PTSD rTMS, $p = 0.005$). PTSD Sham mice explored also significantly less the novel object compared to NS Sham mice ($p = 0.0002$); a similar trend was observed for PTSD Veh mice *vs* NS Veh mice ($p = 0.051$).

Reexposure. Foot-shock conditioning significantly decreased the latency of freezing during reexposure to the context, regardless of treatment ($F(7, 140) = 12.76$, $p < 0.0001$; NS Veh *vs* PTSD Veh, $p < 0.0001$; NS Flx *vs* PTSD Flx, $p = 0.011$; NS Sham *vs* PTSD Sham, $p < 0.0001$; NS rTMS *vs* PTSD rTMS, $p < 0.0001$). Foot-shock conditioning also increased the total duration of freezing ($F(7, 140) = 51.59$, $p < 0.0001$), however, *p*-values and effect sizes were smaller for PTSD Flx mice ($p = 0.0003$, $d = 1.95$ *vs* NS Flx) and PTSD rTMS mice ($p = 0.008$, $d = 2.02$ *vs* NS rTMS) compared to PTSD Veh mice ($p < 0.0001$, $d = 3.79$ *vs* NS Veh) and PTSD Sham mice ($p < 0.0001$, $d = 3.09$ *vs* NS Sham). Fluoxetine and chronic rTMS decreased the total duration of freezing in stressed mice (PTSD Veh *vs* PTSD Flx, $p < 0.0001$; PTSD Sham *vs* PTSD rTMS, $p < 0.0001$). No differences were observed between NS mice and thus for the two behavioral measures (Fig. 3B).

Chronic rTMS acted on a distributed network distant from stimulation site

Prefrontal regions. Chronic rTMS treatment significantly increased c-Fos densities in the IL for PTSD mice only ($F[7,45] = 6.62$, $p < 0.0001$; *vs* NS rTMS, $p = 0.001$; *vs* PTSD Sham, $p = 0.001$; *vs* PTSD Flx, $p = 0.001$). Otherwise, PTSD mice did not differ from NS mice, regardless of treatment (Fig. 4A). In the PrL, no differences were found between groups ($F[7,45] = 1.10$, $p = 0.38$; Table 1). Chronic rTMS treatment significantly increased c-Fos densities in the Cg for NS mice ($F[7,45] = 5.14$, $p = 0.0004$; NS Sham *vs* NS rTMS, $p = 0.017$; NS Flx *vs* NS rTMS, $p = 0.046$). PTSD rTMS mice displayed higher densities than PTSD Flx mice ($p = 0.029$) but were no statistically different from PTSD Sham mice ($p = 0.22$), although the effect size was $d = 1.54$ in favor of PTSD rTMS (Fig. 4A).

Amygdala. Foot-shock conditioning decreased c-Fos densities in the BLA ($F[7,45] = 17.62$, $p < 0.0001$; NS Veh *vs* PTSD Veh, $p = 0.004$; NS Sham *vs* PTSD Sham, $p = 0.001$), but was prevented by fluoxetine (PTSD Veh *vs* PTSD Flx, $p = 0.006$) and rTMS (PTSD Sham *vs* PTSD rTMS, $p = 0.045$). Concurrently, we observed higher c-Fos densities in NS Flx mice when compared to any other group (*vs* NS Veh, $p < 0.0001$; *vs* NS rTMS, $p < 0.0001$; *vs* PTSD Flx, $p < 0.0001$), while rTMS had no effect in NS mice (NS Sham *vs* NS rTMS, $p = 0.41$; Fig. 4B). In the central amygdala, only NS Flx mice had lower c-Fos densities ($F[7,45] = 2.42$, $p = 0.038$; *vs* NS Veh, $p = 0.014$), while other groups displayed similar densities (Table 1).

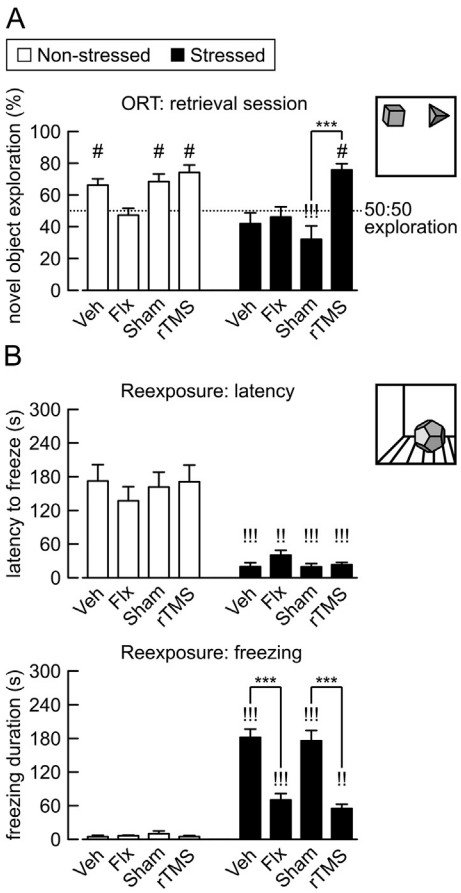

Fig. 3. Behaviors. (A) Object recognition task. The dot-line indicates theoretical mean at 7.5 s. (B) Up: latency to freeze during reexposure. Bottom: freezing duration during reexposure. Symbols: against theoretical 7.5 s #$p < 0.0001$; Stressed (PTSD) *vs* Non-Stressed (NS) ! $p < 0.05$, !! $p < 0.01$, !!! $p < 0.001$; treated *vs* un-treated $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Hippocampus. No differences were detected in the dCA1 ($F[7,45] = 0.737$, $p = 0.64$; Table 1). In the vCA1, c-Fos densities were increased for PTSD Flx mice ($F[7,45] = 79.98$, $p < 0.0001$; *vs* PTSD Veh, $p < 0.0001$) and PTSD rTMS mice (*vs* PTSD Sham, $p < 0.0001$). PTSD Flx mice also displayed higher c-Fos densities compared to PTSD rTMS mice ($p < 0.0001$). In PTSD mice, both treatments increased c-Fos densities when compared to NS mice (NS Flx *vs* PTSD Flx, $p < 0.0001$ and NS rTMS *vs* PTSD rTMS, $p < 0.0001$, respectively). While fluoxetine increased c-Fos densities regardless of the stress condition (NS Veh *vs* NS Flx, $p < 0.0001$; NS Flx *vs* NS rTMS, $p < 0.0001$), chronic rTMS had no effect in NS mice (NS Sham *vs* NS rTMS, $p = 0.934$; Fig. 4C).

Discussion

Focal targeting of the vmPFC with rTMS was achieved for the first time in a mouse strain through stereotaxic framing. The current study confirmed that a suprathreshold, high-rated rTMS sequence could directly activate a cortical target with limited spatial inaccuracy. Although several key prefrontal structures were on the path of the repeated electromagnetic fields, a unique 0.5 mm^2 area was significantly modified on c-Fos analysis, overlapping subunits of IL and ventral-most PrL cortices that functionally relate to the vmPFC in humans [51]. Nevertheless, the

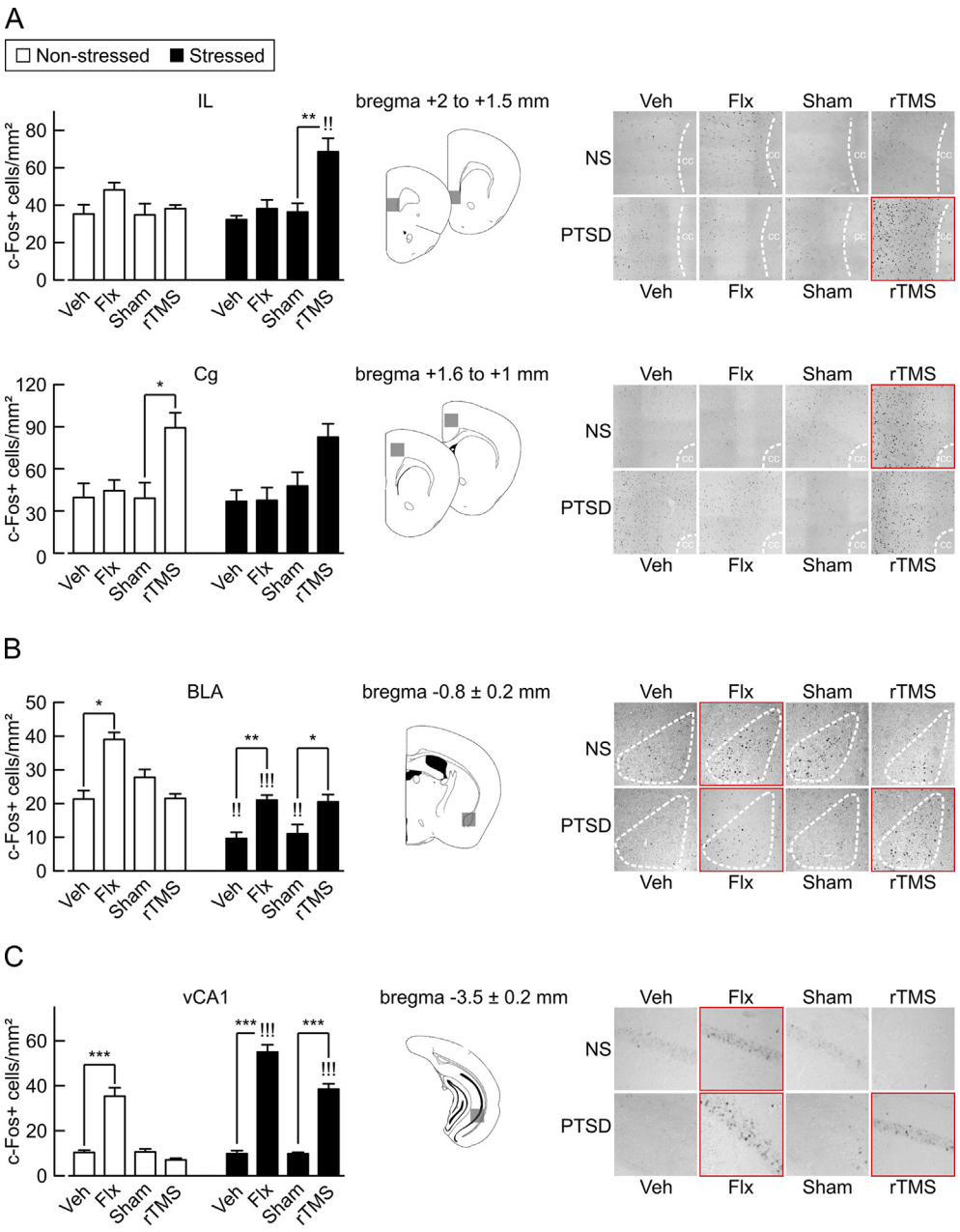

Fig. 4. Reexposure-induced c-Fos densities. (A) PFC scores (IL and Cg). (B) BLA scores. (C) vCA1 scores. Representative micrographs were chosen from the analysis range (red-outlined: significant). Dashed outlines delimit either the forceps minor of the corpus callosum (cc) or the BLA. Symbols: Stressed (PTSD) *vs* Non-Stressed (NS) ! $p < 0.05$, !! $p < 0.01$, !!! $p < 0.001$; treated *vs* un-treated *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cumulated effect of 750 magnetic pulses iterated at 12 Hz could have a broader range on-site, dimmed on c-Fos IHC by immediate inter-neural GABAergic regulation, as biphasic pulses are described to act synchronously on multiple neuron populations [58]. Because c-Fos expression appears in reactive neurons regardless of their function (glutamatergic or GABAergic), the spatial range of One-session rTMS might be accurately reflected by the topographical maps, while the functional range remains undefined in the current study. The second component of the study applied a 5-session chronic rTMS treatment to the vmPFC in a mouse model of PTSD,

Table 1
Normalized c-Fos densities post-reexposure (c-Fos + cells/mm^2). PrL: prelimbic cortex; CeA: central amygdala; dCA1: dorsal CA1; PTSD: post-traumatic stress disorder; Veh: vehicle; Flx: fluoxetine; rTMS: repetitive transcranial magnetic stimulation. Data shown as mean ± SEM; *$p < 0.05$; bold text shows significant differences (treated *vs* untreated).

	Non-stressed				PTSD				1-way ANOVA	
	Veh	*Flx*	*Sham*	*rTMS*	*Veh*	*Flx*	*Sham*	*rTMS*	*F*	*p*
PrL	15.2 ± 2.4	13.2 ± 2.7	9 ± 1.5	9.5 ± 2	11.8 ± 2.6	14.8 ± 1.2	10.8 ± 2.7	11.7 ± 1.9	1.10	ns
CeA	7 ± 0.8	**2.6 ± 0.2**	4 ± 0.8	5.5 ± 0.8	4.2 ± 0.9	5.5 ± 1	5 ± 1.1	5.2 ± 0.6	2.42	*
dCA1	2.8 ± 0.4	1.8 ± 0.5	1.8 ± 0.4	2.7 ± 0.6	2.3 ± 0.5	2.2 ± 0.2	2 ± 0.6	2 ± 0.4	0.74	ns

which prevented the occurrence of behavioral impairments in mnesic performance and extinction dynamics while acting on associated activity patterns in the IL, the BLA and the vCA1.

Episodic-like memory impairments were reported in stressed mice before [24] and confirmed in the present study. The results show that chronic rTMS treatment fully reversed the stress-induced cognitive impairment at a mid-to-long-term temporal range, as the last session of stimulation was undertaken six days prior to the retrieval phase of the ORT. Preserved mnesic capacities were seen during reexposure to context at day 22, where the latency to display freezing was significantly shorter than NS rTMS mice and no different from PTSD Sham mice. However, extinction dynamics were enhanced: within the 300-sec timeframe of behavioral analysis, a significant decrease of freezing duration was observed in PTSD rTMS mice, supporting the role of chronic rTMS treatment to the vmPFC as a valid option for acting on fear neurocircuits. Recently, it has been shown that a single 30-min isoflurane anesthesia could decrease depressive-like behaviors in a rodent model of learned helplessness [59]; however, no behavioral modifications appeared in PTSD Sham mice when compared to PTSD Veh mice. Fluoxetine treatment did not improve memory performance and decreased the baseline ORT score in both NS and PTSD Flx mice. To our knowledge, the occurrence of a fluoxetine-induced short-term memory impairment is paradoxical in this experimental paradigm [60,61], although similar observations were previously reported: chronic fluoxetine treatment (10 mg/kg) was associated to interferences with the MAP/ERK2 memory pathway, which could relate to long-term memory impairments in Swiss mice for a similar ORT design [62,63]. Such discrepancies might stem from the variability of protocols employed, using different administration methods (e.g. intraperitoneal *vs per os*), doses (e.g. 10 mg/kg *vs* 15 mg/kg) and periods (e.g. acute *vs* chronic). Because short-term memory performance was also decreased in NS Flx mice, the behaviors of PTSD Flx mice might not reflect a deleterious effect of the compound on memory retrieval, but a lack of motivational drive to perform this particular task. To further support this view, we observed that long-term memory of the foot-shock context was preserved in the PTSD Flx group, which displayed a latency to freeze similar to the PTSD Veh group during reexposure. Extinction dynamics were also enhanced by fluoxetine, which confirms the beneficial aspect of the drug in this model of PTSD.

Despite similar behaviors during reexposure to the context, rTMS-treated and fluoxetine-treated mice displayed different c-Fos densities. While c-Fos was directly associated to the reexposure, it permitted to probe indirectly the long-term modifications in brain reactivity induced by the treatments. Chronic rTMS increased significantly c-Fos densities in the IL for PTSD mice. However, no direct effect of foot-shock conditioning was observed on the IL reactivity. Because the main role of the IL consists in top-down fear extinction, the low c-Fos densities observed in NS mice were expected, while low c-Fos densities observed in PTSD Veh and PTSD Sham mice correlated with high amounts of freezing during reexposure [64]. PTSD Flx mice displayed similar c-Fos densities to PTSD Veh in the IL, which suggests that the enhancement of extinction dynamics was not correlated to top-down control over the amygdala. Cellular densities in the PrL did not differ between groups, which was expected due to the role of this region in fear conditioning, and not extinction [64]. Cellular densities in the Cg were only increased by rTMS in NS mice, even though a qualitative increase was found for PTSD rTMS mice. A recent study showed that a high-frequency rTMS treatment in a rat model of PTSD ameliorates symptoms by reversing impaired glutamate signaling in the Cg [31]. High-rated rTMS was already described to provoke lasting plastic effects [65,66]; in our study, c-Fos densities in the Cg were still impacted by rTMS 10 days after the last treatment session, suggesting that such mechanisms could be at play in the behavioral improvement of PTSD rTMS mice.

The projections of prefrontal structures to the BLA are paramount in the pathophysiology of PTSD [67]. The absence of fluoxetine-induced effects on prefrontal structures, despite equivalent behavioral outcomes on the hallmark freezing behavior, supposes that different neurobiological mechanisms are at play between rTMS and fluoxetine. Both treatments reversed stress-induced low c-Fos densities observed in the BLA. Though amygdala overdrive relates to fear expression, the BLA mediates the acquisition of fear extinction learning [68] and the expression of extinction memory via the intercalated neurons and their inhibitory effect on the central amygdala [69]. The c-Fos staining did not differentiate types of neurons, and favoring one microcircuit over another (e.g. intercalated neurons) could correlate to decreased freezing [5]. Higher c-Fos densities in the BLA could thus relate to increased extinction dynamics observed for PTSD Flx and PTSD rTMS mice. The effects of fluoxetine were also visible in NS mice, which displayed higher c-Fos densities in the BLA, while NS rTMS mice did not differ from NS Sham mice. The fluoxetine treatment also modified c-Fos densities in the central amygdala, an effect that was not obtained for any other groups, and which suggests a baseline effect of fluoxetine on the amygdala formation. Unlike fluoxetine, chronic rTMS might work on dedicated cortico-subcortical pathways (e.g., vmPFC to BLA), as modifications in the BLA were only observed for PTSD rTMS mice. Finally, both treatments affected the vCA1 in PTSD mice while having no effect in the dCA1. The functional projection from vCA1 to PFC is specifically involved in cognitive processes (e.g. working memory) and context-dependent emotional regulation (e.g. fear extinction), and can be disrupted in neuropsychiatric disorders [8], including PTSD. The vCA1 projects monosynaptically on both the vmPFC and the BLA and its excitability was previously shown reduced in the same PTSD model, which was reversed by SSRI treatment [60]. NS Flx mice displayed significantly higher c-Fos densities in the vCA1 compared to NS Veh mice, while NS rTMS mice did not differ from NS Sham mice, which further underlie the different mechanisms of action of the treatments. Because fluoxetine can act on neurogenesis in the dentate gyrus of the hippocampus, the treatment could have produced more plastic, long-term modifications that were not fully present at the time of the ORT, although this analysis was beyond the scope of the study Overall, the lack of c-Fos reactivity in PTSD Veh and PTSD Sham mice was not consistent with previous literature [24], but such delay between foot-shock conditioning and reexposure was not studied before.

Conclusions

For the first time in mice, we have shown that chronic rTMS treatment of the vmPFC reversed pathological behaviors while acting specifically and with economy (few effects of rTMS alone) on distributed networks of fear neurocircuitry. These functional modifications were direct for PFC structures and stemmed indirectly to the BLA and vCA1, generating lasting, distant effects that self-sustained up to ten days after the last treatment session. The strengthened functional connectivity between the vmPFC and the BLA could be of prime relevance if paired with exposure therapy in humans by providing sustained extinction of fear traces.

Conflicts of interest

The authors declare having no conflicts of interest. No financial support was received that could have influenced its outcome.

Acknowledgements

This work was funded by a thesis grant for Marc Legrand. Hardware was acquired through a support from the Région Centre (France; 00085204).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.brs.2018.09.003.

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