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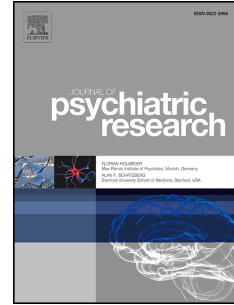
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-Title Page-

rTMS mechanisms for posttraumatic stress disorder treatment in a mouse model.

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-Abstract-

Background: Posttraumatic stress disorder (PTSD) is a psychiatric disease that may follow traumatic exposure. Current treatments fail in about 30% of patients. Although repeated transcranial magnetic stimulation (rTMS) applied to the prefrontal cortex has been shown to be effective in the treatment of PTSD, the mechanisms need further investigation.

Objective: Using a PTSD animal model, we verify the beneficial effect of rTMS, and explore the changes it induces on two putative PTSD mechanisms, GABA/ glutamate neurotransmission and neuroinflammation.

Methods: PTSD-like symptoms were elicited in twenty-six mice using a foot-shock conditioning procedure. Fourteen of the 26 were then treated using rTMS (12 were untreated). In the control group ($n=30$), 18 were treated with rTMS and 12 were untreated. Animals were sacrificed after re-exposure. The infralimbic (IL) cortex, basolateral amygdala (BLA) and ventral CA1 (vCA1) were isolated using laser microdissection. mRNA was then investigated using PCR array analysis targeting GABA/ glutamate and inflammatory pathways.

Results: The rTMS treatment significantly decreased the contextual fear memory phenotype. These changes were associated with reduced mRNA expression related to inflammation in the IL cortex and the vCA1, and lowered mRNA-related glutamate neurotransmission and increased GABA neurotransmission in the BLA.

Conclusion: Our results suggest that our rTMS treatment was associated with local anti-inflammatory effects and limbic effects, which seemed to counteract PTSD effects. Several of these changes (both stress- and rTMS-induced) have implications for the drug sensitivity of limbic brain areas, and may help in the design of future therapeutic protocols.

Keywords: PTSD, rTMS, neuroinflammation, GABA, glutamate

Introduction

Posttraumatic stress disorder (PTSD) is a psychiatric disease that may follow a traumatic exposure to stress, for example, one's own or another's death. While lifetime prevalence in the general population is 0.5–4% [1], it is higher in at-risk populations, such as soldiers [2, 3]. Symptoms are characterized by persistent fear reactions that include reviviscence, flashbacks, nightmares, hyperarousal, and cognitive disorders. Although not completely understood, the psychopathology of PTSD is centered on amygdala hyperactivity, frontal cortex hypoactivity, and impaired emotional memory [1]. Current treatment combines psychotherapies and pharmacotherapies; however, this fails in about 30% of patients [4]. More efficient treatments are needed.

One treatment target is to improve fear memory extinction. This can be achieved by the application of an extinction procedure after 24h latency [5], and involves the ventromedial prefrontal cortex (vmPFC), equivalent to the infralimbic (IL) cortex in rodents [6], and the basolateral amygdala [5, 7]. The ventral hippocampus, notably the ventral CA1 (vCA1) is another important region to consider, as it projects to both the IL cortex and the basolateral amygdala (BLA) [8], which play a role in emotional information management [9]. Therefore, targets for extinction could be a decrease in BLA activity [10], increased IL cortex activity [10], and increased vCA1 activity [11].

Among emerging PTSD treatments, repeated transcranial magnetic stimulation (rTMS) is a non-invasive method that consists in applying a magnetic field, using a coil placed above the scalp in front of the brain region of interest. The focused magnetic field leads to neuronal depolarization in the targeted structures, influencing, in turn, brain connectivity [12]. FDA approval was given to use rTMS as a treatment for major depressive disorder in 2008. However, its effects in people with PTSD need to be confirmed, despite studies showing its efficiency [12]. Specifically, it has been demonstrated that when rTMS targets the vmPFC and the dorsolateral PFC (both structures are hypoactive in PTSD, and involved in the suppression of fear responses [13]) PTSD symptoms improve [12]. However, the frequency of stimulation is important to consider, since high-frequency stimulation has been found to be more effective than low-frequency stimulation [12].

Studies have shown that rTMS has long-lasting effects [14], which suggests that the induced neuronal depolarization may be associated with brain restructuration that may, in turn, involve the GABA/ glutamate balance. Alteration to the latter is a physiopathological hypothesis of PTSD [15], and normal fear extinction is related to balanced GABA/glutamate neurotransmission in the mPFC-amygdala circuit [16]. The complex equilibrium underpinning fear extinction combines maintained (but not overactive) GABA tone, together with steady glutamatergic activity [16].

Furthermore, rTMS has been found to have beneficial anti-inflammatory and antioxidant effects [17, 18], and neuroinflammation is another physiopathological hypothesis of PTSD [19], as central and peripheral inflammation, including activation of autoimmune cells and cytokine production [20], is observed in patients. In this context, preclinical studies show that

pro-inflammatory cytokines are released in central and peripheral tissues after the activation of central fear circuits [20]. PTSD is associated with a breakdown in the compartmentation of central and peripheral areas, the migration of peripheral monocytes to the brain, and a subsequent increase in microglia activity [20, 21]. Circulating monocytes infiltrate the brain, and differentiate into activated M1 macrophages, which may change, over time, into anti-inflammatory M2 phenotypes [22]. Whether rTMS can influence the efficacy of these phenomena is not known.

Against this background, our objectives were: (i) to verify the beneficial effect of rTMS in a PTSD animal model; and (ii) to explore change in GABA/ glutamate and neuroinflammation after rTMS brain stimulation.

Methods

Animals

Fifty-six male Swiss mice (8 weeks old at arrival, Janvier Labs SAS, France) were used in this study. Animals were given one week to become accustomed to the facility's conditions (21°C, 12h/12h light-dark cycle with lights on at 7:30 AM). They had free access to food and tap water.

They were then randomized into four experimental groups. Twenty-six mice were subjected to foot-shock conditioning to induce PTSD-like symptoms (Stress, $n=26$). Thirty other animals were used as a control, and were not exposed to the foot-shock (Control, $n=30$). In the Stress group, one subgroup was exposed to rTMS (rTMS-Stress, $n=14$), while the other group was not (No-rTMS-Stress, $n=12$). Similarly, in the Control group, 18 animals were exposed to rTMS (rTMS-Control, $n=18$), and 12 were not (No-rTMS-Control, $n=12$). The experimental time course is shown in Supplementary Figure 1.

All experimental procedures were carried out in accordance with the ethical standard defined by the French National Institute of Health and Medical Research, and European Community guidelines for the care of laboratory animals (no. 86/ 609), and were approved by CEEA Val de Loire N°19 under number #19455-2019021816208110_v4.

Animal conditioning

Experimental design

After spending one week in the laboratory facility, animals in the Stress group were exposed to the conditioning (exposure (EXP), D1). Then, they were exposed to five rTMS sessions (D7–D11). Twenty-one days after EXP, animals in the Stress group were re-exposed to the same conditioning context (re-exposure (RE-EXP), D22).

Exposure protocol

As described in earlier work [23], PTSD-like symptoms were induced by a short, but very intense exposure to stress on day 7. Each mouse was individually placed in a semi-transparent cage (half of the walls were blinded) which contained an unknown object (a miniature, but heavy human skull, referred to as the traumatic object). The cage was small (18 x 15 x 13 cm) to allow a rapid and complete exploration of the environment, and its floor was made of metal barrels that conducted electrical current. The mouse was allowed to freely explore the cage and the traumatic object for 190 sec (Baseline). Baseline locomotor activity was measured to ensure that animals in all groups behaved similarly. Then, two electric shocks were applied (stress conditioning, intensity 1.5 mA, duration 2 sec each, with a 6 sec interval). Then, the mouse remained in the cage for 60 additional seconds before being returned to its home cage in the animal facility. Non-stressed animals were submitted to the same procedure, but without the electric shock.

Re-exposure protocol

PTSD-like symptoms were assessed 21 days after stress conditioning, as follows. Both Stress and Control mice were re-exposed (for 300 sec) to the same context, including the traumatic object placed in the same position as before, but with no electric shock.

Behavior analysis

In both experimental conditions (EXP and RE-EXP), behavior was videorecorded for off-line scoring. Fear expression was scored using behavioral markers, such as slow movements (moving the head but not the body) or immobilization (freezing). The manual analysis was performed by an experienced specialist using ethological keyboard software (Solomon Coder) with time resolution set at 200 ms. Since Baseline and RE-EXP periods were not of the same duration, results were expressed as a percentage of the total duration.

Magnetic stimulation procedure

The rTMS device

The device is the same as the one described in previous work (MagVenture, A/S, Denmark) [23]. Coil specifications (Cool-40, 40 mm circular coil for rodents) were in accordance with those described in Parthoens et al. [24]. At maximum mean output (190A/ μ sec), the stimulator (MagPro R30) induces an electrical field estimated to be 100V/m at 5 mm depth under the brain surface.

Coil positioning

Animals were anesthetized under isoflurane. They were then placed on their ventral side, with their head fixed in a stereotactic frame with atraumatic auxiliary ear bars. The targeted brain volumes included cingulate (rostral part), prelimbic (PrL) and infralimbic (IL) cortices. The stimulation rTMS coil was positioned tangential to the skull, and centered on the interhemispheric line, at Bregma +2 mm, to ensure bilateral stimulation. To enhance the spatial precision of the stimulation, and minimize the magnetic field's reach, we employed a custom-made amagnetic manipulator specifically designed to handle the coil, and a protective shield (0.5 mm thick, nickel/ iron alloy; MagnoShield FLEX+, Aaronia) positioned over the caudal part of the head, starting at Bregma +1 mm as detailed previously [23].

Stimulation parameters for rTMS

Parameters mimicked the repetitive protocols used in humans for neuropsychiatric applications. In humans, significant effects have been obtained at two intensities, below (80–90%) and above (105–120%) the motor threshold, and 115% of the mean motor threshold stimulation has been found to preferentially target neurons rather than interneurons [23]. The motor threshold was previously determined in another cohort of mice of the same age and weight, in order to not alter the brain parameters of the cohort considered in this study before starting the experiment. Motor threshold recordings were previously published by our team

[23]. These parameters were used for all animals (115%, 84A/ μ sec and 44% of the mean output of the stimulator). Other stimulation parameters were set as follows: magnetic pulse train frequency: 12 Hz, 25 trains of 30 pulses delivered with a 14 sec-interval, making a total of 750 pulses per session for a total duration of around 7 minutes.

rTMS stimulation protocol

rTMS was used as an early treatment following conditioning. The protocol consisted of five sessions scheduled from D8 to D12, with 24-hour intervals between sessions, resulting in a total of 3750 magnetic pulses. This approach has been documented to alleviate PTSD-like symptoms subsequent to foot-shock conditioning in mice [18]. Animals were stimulated between 9 AM and 5 PM, at fixed times, and in the same order, to ensure 24h intervals between sessions. Control mice underwent anesthesia during the corresponding period of each day for an equivalent duration.

Brain analysis

Brain removal procedure

On Day 23 (two days after RE-EXP), all mice were sacrificed. Sacrifice was conducted two days after the final behavioral test, to mitigate potential immediate early gene transcription influences that might arise from re-exposure. Cervical dislocation was used to prevent any interference with lethal drug administration during further biochemical analyzes. The brain was removed rapidly, with the head placed over an ice bed. Immediately after removal, it was immersed in a cold isopentane solution (-55°C) for instant freezing. All brains were stored at -80°C until microdissection.

ROI (Region Of Interest) cutting (laser microdissection procedure)

Brains were cut into slices of 20 μm , using a cryostat (MMFrance, Cryostar NX70). Slices of interest were placed on PEN-membrane slides (Leica, 11505158). Slides were then colored using a bath of 1% Cresyl Violet Acetate (Merck, C5042-10G) followed by a wash bath of 70% ethanol (Merck, 51976-500ML-F). The infralimbic cortex (IL), basolateral amygdala (BLA) and ventral CA1 (vCA1) were cut using a laser microdissector (Leica, LMD 7000), according to the mouse brain atlas [25]. For each mouse the majority of each ROI were cut (IL 10 slices \pm 2, BLA 13 slices \pm 1, vCA1 8 slices \pm 1). For each ROI and for each mouse cuts were pooled. Cut areas fell under gravity into the lysis buffer of the RNA extraction kit (Macherey Nagel, NucleoSpin RNA XS) just before freezing at -80°C , and until RNA extraction.

RNA extraction

RNA extraction followed the manufacturer's recommendations (Macherey Nagel, NucleoSpin RNA XS). RNA quality (RIN: 7.1 ± 0.04) and quantity were evaluated using the 4200 TapeStation (Agilent, High Sensitivity RNA ScreenTape).

RT² Profiler PCR array test

For each mouse, total RNA were retrotranscribed using the RT² First Strand kit (Qiagen). The resulting cDNA was divided into two parts and quantified using two real-time RT² Profiler PCR arrays performed simultaneously (for the GABA/ glutamate pathway: PAMM-152ZG-4, and for the inflammatory pathway: PAMM-077ZG-4 384 well plates) using RT² SYBR[®] Green qPCR Mastermix. Each sample was tested in singlicate. Targets with undetectable threshold cycle (Ct) values were considered missing data. Only samples passing all three controls (human genomic contamination, reverse transcription control, and positive PCR control) were included in subsequent analyses. Quantification cycles were determined using the second derivative maximum method. For each plate, five reference genes were tested (beta-actin (Actb), beta-2-microglobulin (B2m), glyceraldehyde-3-phosphate (Gapdh), glucuronidase beta (Gusb), heat shock protein 90 alpha family class B member 1 (Hsp90ab1)). Using the geNorm software package, the most stable reference genes were identified for each array. Briefly, this validated algorithm determined the minimal number of the most stable reference genes giving a pairwise variation below a validated threshold of 0.15 [26]. For each array, Actb and Hsp90ab1 were sufficiently stable for normalization according to the geNorm algorithm. 2- Δ CT method was calculated using mean of samples as a calibrator, then relative expressions were calculated by dividing the 2- Δ CT of target gene expression by the geometric mean of the two previously described reference genes [27]. Any relative expressions exceeding the 95% confidence interval were considered outliers. Their exclusion takes into account the multiple potential artifacts that could be induced by the large number of steps in the technique. Targets for which more than half of the sample was not detectable were considered as non-analyzable. The 160 resulting targeted genes, and their descriptions are presented in Supplementary Tables 1–6 and 7–8, respectively.

Statistical analyses

MATLAB software (MathWorks, r2019a) was used for all statistical analyses. Normality was checked with the Kolmogorov–Smirnov test for all groups, and for all variables. Behavioral variables were analyzed with a two-way, repeated measures analysis of variance (ANOVA). Means for the four groups (No-rTMS-Control, rTMS-Control, No-rTMS-Stress, rTMS-Stress) were compared at the two experimental times (Baseline *vs.* RE-EXP). The effect of stress, treatment, time, and their interaction (stress \times treatment \times time) were considered. Molecular biology variables were analyzed using a two factor ANOVA. The two factors were the stress condition (No-Stress *vs.* Stress) and the treatment (No-rTMS *vs.* rTMS). For each analysis, the effect of stress, treatment, and their interaction (stress \times treatment) were considered.

For all analyses, when ANOVAs revealed a significant effect, *post hoc* Scheffé comparisons were performed for all pairs. This test was chosen due to its high robustness and suitability for mRNA comparisons. Notably, the sample size for each mRNA target may differ due to outliers and undetectable transcripts. The significance threshold was set at $p < 0.05$, and data are expressed as mean \pm standard error of the mean (SEM).

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Results

Behavior

Percentage of time devoted to slow movement/freezing

Locomotor activity was measured at Baseline and RE-EXP. As shown on Figure 1, behavior at Baseline was the same for all four groups, but not during RE-EXP (interaction: $F(1,52)=10.14$ $p<0.01$). At RE-EXP, the No-rTMS-Stress group exhibited a higher level of inactivity compared to the No-rTMS-Control group (*post hoc* test $p<0.01$), suggesting a stress effect, and memorization of fear and its context. The No-rTMS-Stress group was also characterized by a higher level of inactivity than both rTMS-Control and rTMS-Stress groups (*post hoc* test $p<0.01$ for both). Conversely, the rTMS-Stress group did not significantly differ from either the rTMS-Control or the No-rTMS-Control group (*post hoc* tests $p=0.19$; $p=0.09$, respectively).

Molecular biology

Only pertinent outcomes demonstrating a significant effect along with significant *post hoc* findings are reported here, as all other results are detailed in Supplementary Tables 1–6.

GABA/glutamate pathway mRNA analysis

Our analysis found that in the IL cortex (Figure 2.A–D; Supplementary Table 1), Stress exposure was associated with increased mRNA coding for the glutamate ionotropic kainate Grik5 ($p<0.05$ Figure 2.A), the metabotropic Grm8 ($p<0.05$ Figure 2.B) receptor, and the postsynaptic protein Dlg4 ($p<0.05$ Figure 2.C). The rTMS treatment was associated with decreased Nsf coding ($p<0.05$ Figure 2.D), which is a regulator of vesicular dependent protein membrane trafficking. No interaction (stress \times treatment) was observed.

No significant effect of stress was observed in the BLA, (Figure 3.A–L; Supplementary Table 2). The rTMS treatment increased mRNA coding for glutamate ionotropic AMPA Gria1 ($p<0.05$ Figure 3.A) and metabotropic Grm7 ($p<0.05$ Figure 3.B) receptors, the GABA transporter Slc6a13 ($p<0.05$ Figure 3.C), the following GABA A receptors: Gabra1 ($p<0.05$ Figure 3.D), Gabra5 ($p<0.05$ Figure 3.E), Gabrg3 ($p<0.05$ Figure 3.F), and Adcy7 adenylyl-cyclase, an enzyme involved in the downstream signaling of glutamate and GABA receptors ($p<0.05$ Figure 3.G). The treatment also decreased mRNA coding for the GABA B receptor Gabbr2 ($p<0.01$ Figure 3.H), the glutamate ionotropic kainate receptor Grik5 ($p<0.05$ Figure 3.I) and the glutamate transporter Slc17a7 ($p<0.05$ Figure 3.J). Interactions (stress \times treatment) were observed for mRNA coding the GABA A receptor Gabra2 (ANOVA: $p<0.05$ Figure 3.K) and the metabotropic receptor Grm5 (ANOVA: $p<0.01$ Figure 3.L), with both showing an increase in rTMS-Control comparatively to No-rTMS-Control ($p<0.05$).

In the vCA1 (Figure 4.A–I; Supplementary Table 3), mRNA coding was higher in Stressed animals for glutamate ionotropic kainate Grik1 ($p < 0.05$ Figure 4.A) and metabotropic Grm5 ($p < 0.01$ Figure 4.B) receptors, and the GABA A receptor Gabra4 ($p < 0.05$ Figure 4.C). Conversely, coding was lower for the glutamate transporter Slc17a7 ($p < 0.05$ Figure 4.D). rTMS-treated animals were characterized by higher mRNA coding for GABA A receptors Gabra2 ($p < 0.05$ Figure 4.E), the GABA transporter Slc6a1 ($p < 0.05$ Figure 4.F) and the purinergic receptor P2rx7 ($p < 0.05$ Figure 4.G). Conversely, coding was lower for the calcium channel Cacna1a ($p < 0.05$ Figure 4.H), and the glutamate metabotropic receptor Grm1 ($p < 0.01$ Figure 4.I). No interaction was observed.

Inflammatory pathway mRNA analysis

In the IL cortex (Figure 5.A–D; Supplementary Table 4), stress induced an increase in mRNA coding for regulators of the inflammatory response: the interleukin 1 receptor accessory protein Il1rap ($p < 0.05$ Figure 5.A) and the Toll-like receptor Tlr2 ($p < 0.05$ Figure 5.B). The rTMS treatment was associated with decreased coding for the inducible nitric oxide synthase Nos2 ($p < 0.05$ Figure 5.C). The mRNA level was lower for the cytokine receptor Il6-ra in the rTMS-Stress compared to the No-rTMS-Stress condition (interaction, $p < 0.05$ Figure 5.D).

In the BLA (Figure 6.A–D; Supplementary Table 5), Stress increased coding of the chemokine Ccl11 ($p < 0.05$ Figure 6.A). The rTMS treatment decreased coding for the colony-stimulating factor Csf1 ($p < 0.05$ Figure 6.B), and increased coding for the Toll-like receptor Tlr3 ($p < 0.05$ Figure 6.C). Interaction (stress \times treatment) was observed for mRNA coding the chemokine Ccr3 (ANOVA: $p < 0.01$ Figure 6.D) showing an increase in rTMS-Control comparatively to No-rTMS-Control and rTMS-Stress groups ($p < 0.05$ for both).

In the vCA1 (Figure 7.A–G; Supplementary Table 6), Stress was followed by increased mRNA coding for the cytokine receptor alpha Il6-ra ($p < 0.01$ Figure 7.A), and the nuclear transcription factor Fos ($p < 0.01$ Figure 7.B), and decreased coding for the transcription repressor Bcl6 ($p < 0.05$ Figure 7.C). The rTMS treatment was followed by increased coding for the integrin Itgb2 ($p < 0.05$ Figure 7.D), but decreased coding for lymphotoxin B Ltb ($p < 0.05$ Figure 7.E), the cytokine receptor Cxcr4 ($p < 0.05$ Figure 7.F), and the Toll-like receptor Tlr2 ($p < 0.05$ Figure 7.G). No interaction was observed.

Discussion

Our results show that IL-centered rTMS prevents the occurrence of the PTSD-like phenotype after traumatic stress exposure. This effect is associated with change in several GABA/ glutamate and inflammation mRNA expressions in the IL, the BLA, and the vCA1.

Behavioral rTMS effects

Prior to EXP, all animals showed the same exploratory behavior in the new context. During RE-EXP, the percentage of freezing behavior in No-rTMS-Stress animals increased, attesting to the PTSD-like phenotype. This increase was largely blunted by IL-centered rTMS, and this result is in agreement with previous preclinical studies [23]. The effect is likely to stimulate IL cortex neurons as: (i) we used a power of 115% of the motor threshold; and (ii) we centered rTMS on the bilateral IL cortex since such a stimulation has been shown to activate IL cortex neurons [23]. Additionally, we used a protective shield on the caudal part of the head. Such a protective shield has been shown to decrease the magnetic field of rTMS [28]. In our case, the reduction in magnetic field was measured with a Gaussmeter (Toplionace, TD8620) at approximately 49%. In these conditions, we cannot exclude that rTMS stimulates other structures protected by the shield.

Molecular analysis

The three regions of interest (the IL cortex, the BLA, and the vCA1), involved in the fear reaction and its extinction, were precisely cut using laser microdissection. The large panel of mRNA assessments increased the complexity of the analysis [29-31]. The complexity was exacerbated by the decision to assess brain response two days after RE-EXP. We restrict our discussion to genes with a transcription level of at least 50% change either from Control or from No-rTMS groups in function of the comparison (stress effect, treatment effect respectively) [32]. Considering the experimental time course, notably the two-day gap between the final behavioral test and sacrifice, it is likely that observed mRNA changes are indicative of chronic, rather than acute alterations.

Stress effect

In the IL cortex, stress is followed by a long-lasting increase in mRNA coding for the kainate ionotropic glutamate receptor type 2 (Grik5), which suggests enhanced glutamatergic functioning within the area [33]. The role of Grik5 in contextual memory and depressive-like behavior, as suggested by knockout mice studies [34], together with our behavioral observations, suggests the presence of an ongoing pathological mechanism in Stressed animals. The enhanced expression of mRNA coding for TLR2 receptors suggests an inflammatory mechanism with microglial activation [37]. Furthermore, TLR2 receptors in the prefrontal cortex are associated with avoidance following social stress [38] underlining the important role of prefrontal inflammation in fear memory.

The stress-induced inflammatory process also targets the BLA, where *Ccl11* mRNA transcription (coding for a proinflammatory cytokine) is enhanced. Its serum level correlates with PCL scores in patients with PTSD, indicating a relation with symptom severity [40].

Activation of the vCA1 is also long-lasting, indicated by an increase in *Fos* mRNA transcription [41]. This enhanced activity is consistent with its role in contextual fear learning and retrieval [42, 43]. The inflammatory mechanism is also observed in the hippocampus, and is shown by an increase in *Il6ra* mRNA expression. The level of *Il6*, a proinflammatory cytokine, is higher in the blood and the cerebrospinal fluid of humans with PTSD, and limbic brain regions in animals [44, 45]. Furthermore, increased *Il6* in the hippocampus is associated with its impaired functioning [46].

Taken together, our results suggest that stress is followed by long-lasting activation of the IL cortex and the vCA1, together with inflammation that targets all studied areas. Our findings are in agreement with descriptions of PTSD as an inflammatory pathology [47].

rTMS effect

In the IL cortex, rTMS blunted inflammation, shown by a decrease in: (i) *Il6ra* mRNA coding, the alpha receptor of *Il6* (cf. the stress effect); and (ii) *Nos2* mRNA coding for inducible NOS (iNOS), a biomarker of inflammation that increases in the brain following stress exposure [48]. Furthermore, iNOS is involved in the stress-induced anxiety-like phenotype [49] and memory impairment [50].

rTMS effects were also observed in other limbic structures, such as the amygdala and the hippocampus, which were not directly centered by the treatment. These effects suggest that the technique induces a global reorganization of the brain. While this reorganization could be attributed to the magnetic field, since the coil that was used lacked focal precision [24], it could also be influenced by the connectivity of the IL region.

In the BLA, rTMS modified GABA/Glutamate pathway. rTMS was associated with an increased expression of GABA_A subunits $\alpha 1$ and $\alpha 5$ mRNA. These rearrangements may modify drug sensitivity, as GABA_A $\alpha 1$ is characterized by high benzodiazepine affinity [51], anxiety [52], and fear memory [53]. Moreover, the increase in the mRNA level of *Slc6a13* coding for the GABA transporter (GAT2) on neurons and astrocytes suggests increased GABA metabolism [54]. The rTMS treatment also could modified glutamate neurotransmission, as suggested by: (i) increased *Grm7* mRNA, coding for type III mGluR7, a predominantly presynaptic receptor regulating neurotransmission [55]; and (ii) decreased *Grik5* mRNA coding for the kainate ionotropic glutamate receptor [56]. The treatment increased *Adcy7* gene expression, a gene coding for adenylate cyclase (AC), an enzyme involved in the catecholamine signal pathway. Unsurprisingly, AC, together with GABA tone acts to regulate the catecholamine signal. It is therefore involved in addiction [57] and depressive mood, since AC overexpression is related to depression-like behavior in mice, while elevated postmortem levels are reported in depressive patients [58]. *Adcy8*, another AC isoform, has been shown to be associated with dissociative symptoms of PTSD in a Genome-Wide Association Study [59].

Lastly, rTMS may reduce neuroinflammation, as it has been found to increase the transcription of Tlr3 [60], an mRNA coding for the Tlr3 receptor. Tlr3 has been found to trigger the production of neuroprotective mediators by astrocytes [61], and reduce neuronal plasticity and memory retention in the hippocampus [62]. Altogether, these observations suggest that rTMS decreases BLA reactivity by increasing GABA tone, and decreasing glutamatergic tone. Since PTSD is characterized by both a GABA deficit and a hyperactive amygdala [15], rTMS may counteract PTSD mechanisms in the BLA.

In the vCA1, rTMS acts on neuroinflammation as follows: (i) it increases Itgb2 mRNA coding for a β 2-integrin expressed in both M1 and M2-polarized macrophages [63]; and (ii) it decreases Tlr2; both of these changes are compatible with a M2 orientation and lower TLR ligand stimulation [47], characterizing inflammation resolution [64, 65]. Furthermore, rTMS is associated with a decrease in Ltb mRNA coding for lymphotoxin-beta, which induces the inflammatory response [66]. Accordingly, the Ltb level in the hippocampus is negatively correlated with anxiety and the fear response in rats [67]. The rTMS-induced increase in P2rx7 mRNA levels is more evidence of an anti-inflammatory effect, as stimulation of the P2x7 receptor reduces preexisting inflammation [68]. Altogether, the vCA1 mRNA profile observed after rTMS treatment is compatible with an inflammation resolution/ anti-inflammatory profile.

The brain plasticity effect of rTMS was previously reported [69]. This plasticity effect was shown to be mediated by microglial cytokine production, underlying the importance of the cytokine signalling in the connectivity-modifying effects of rTMS [69]. The anti-inflammatory effect of rTMS has been suggested in other studies [70]. Our results suggest that rTMS-induced anti-inflammation is a global mechanism that targets the IL cortex, the BLA and the vCA1. The treatment counteracts two PTSD mechanisms—the GABA/glutamate balance and inflammation—and is associated with effect on animal behavior during RE-EXP. Furthermore, rTMS does not modify one, specific pathway but rather acts on a biochemical network, with the final result being a decrease in inflammation and changes in the GABA/glutamate balance compatible with excitability.

Limitations

The motor thresholds for selecting the intensity of the magnetic field was not assessed in this cohort. It was already determined in a previous cohort similar in age and weight and did not vary according the animals [23]. To insure an efficient intensity, we chose an intensity value at 115% of the previously measured threshold. Since we observed behavioral effects after stimulation, the stimulation would have been efficient, even if behavioral effects can be observed below of the motor threshold, at least in humans [71].

Otherwise, a mu-metal sheet was used to reduce the magnetic field outside the targeted zone. We could not rule out stimulation of structures under the shield as the field reduction was not complete.

The molecular assays were performed in singlicate due to the reduced volume of samples. Consequently, the absence of replicate verification may overlook experimental errors [72, 73].

To avoid biased results, we used three strategies: (i) only samples passing all three molecular biology controls (human genomic contamination, reverse transcription control, and positive PCR control) were included in analyses, (ii) any relative expressions exceeding the 95% confidence interval were considered outliers, (iii) we chose the Scheffé *post-hoc* test, which is known for its high robustness and to reduce type I error comparatively to other *post-hoc* tests [74].

Conclusion

rTMS is an effective treatment to promote fear extinction, as it has effects on mRNA expression that are compatible with an anti-inflammatory effect in the IL cortex, the BLA and the vCA1, associated with a decrease in reactivity in the BLA. Several of these changes (both stress- and rTMS-induced) have implications for drug sensitivity in the limbic brain, and may be of help in the design of therapeutic protocols. Our study is a step toward personalized medicine.

Disclosure statement

Grants from the Délégation Générale à l'Armement (DGA) supported the study. The authors declare no competing interests related to this manuscript. The opinions or assertions expressed herein are the private views of the authors and are not to be considered as official or as reflecting the views of the French Military Health Service.

Author contributions

D.C., A.C., F.C, C.B. and W.E. wrote the manuscript and designed the study. D.C. and A.C. carried out statistical analyses. D.C., A.C., J.T., C.C., V.G., L.B. and R.T. were responsible for data pre-analyses. All authors have read and agreed to the published version of the manuscript.

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Data statement

The data presented in this study are available on reasonable request from the corresponding author.

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Figure captions

Figure 1: Percentage of time spent by animals in slow movement or immobilization at Baseline (i.e., prior to shock, if any) and at RE-EXP. Values (percentage of time devoted to slow movement/freezing) are expressed as mean \pm SEM. Differences were analyzed using a two-way repeated measures ANOVA (stress (Control vs. Stress), treatment (No-rTMS vs. rTMS) and time (Baseline vs. RE-EXP). *Post-hoc* Scheffé test: ***: $p < 0.001$ No-rTMS-Stress vs. No-rTMS-Control group; \$\$\$: $p < 0.001$ No-rTMS-Stress vs. rTMS-Control group; †††: $p < 0.001$ No-rTMS-Stress vs. rTMS-Stress group.

Figure 2: Results of the GABA/glutamate pathway mRNA analysis. Differences in the IL cortex as a function of the two groups (Stress and Control), and treatment (No-rTMS or rTMS). A–C. Stress effect. A. Grik5. B. Grm8. C. P2rx7. ANOVA for the stress effect: *: $p < 0.05$. D. Treatment effect: Nsf. ANOVA for the treatment effect: *: $p < 0.05$. Results are expressed as mean \pm SEM.

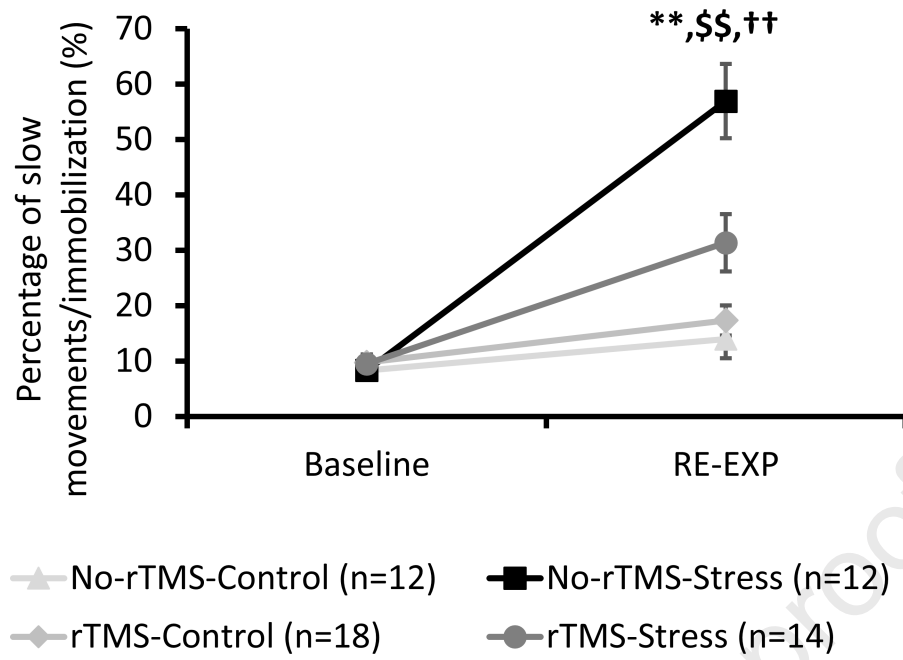
Figure 3: Results of the GABA/glutamate pathway mRNA analysis. Differences in the BLA as a function of the treatment (No-rTMS or rTMS). A–J. Treatment effect. A. Gria1. B. Grm7. C. Slc6a13. D. Gabra1. E. Gabra5. F. Gabrg3. G. Adcy7. H. Gabbr2. I. Grik5. J. Slc17a7. ANOVA for the treatment effect: *: $p < 0.05$; **: $p < 0.01$. K–L. Interaction effect. K. Gabra2. L. Grm5 as a function of group. *: $p < 0.05$ using the *post hoc* Scheffé test. Results are expressed as mean \pm SEM.

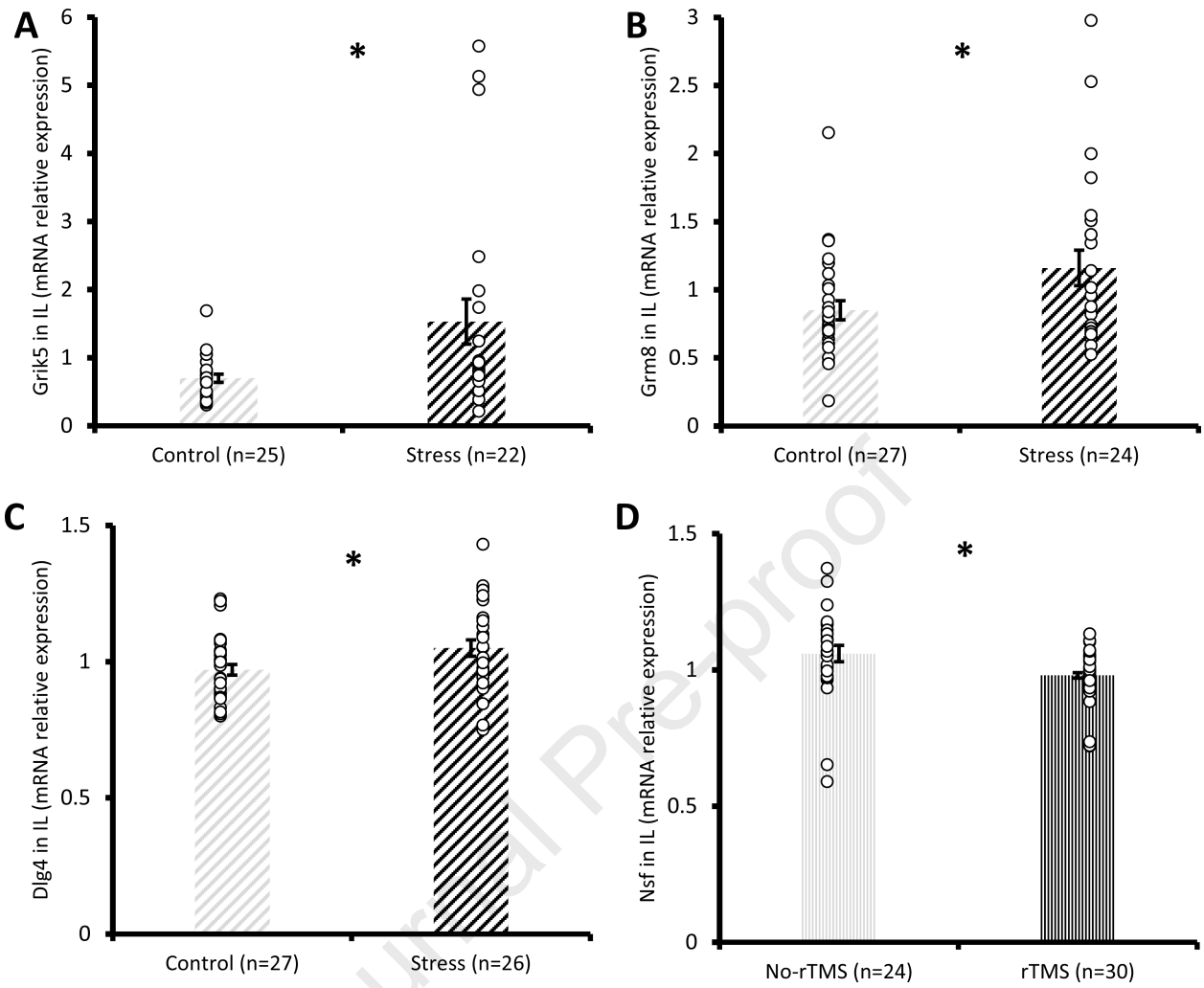
Figure 4: Results of the GABA/glutamate pathway mRNA analysis. Differences in the vCA1 as a function of the two groups (Stress and Control), and treatment (No-rTMS or rTMS). A–D. Stress effect. A. Grik1. B. Grm5. C. Gabra4. D. Slc17a7. ANOVA for the stress effect: *: $p < 0.05$; **: $p < 0.01$. E–I. Treatment effect. E. Gabra2. F. Slc6a1. G. P2rx7. H. Cacna-1a. I. Grm1. ANOVA for the treatment effect: *: $p < 0.05$; **: $p < 0.01$. Results are expressed as mean \pm SEM.

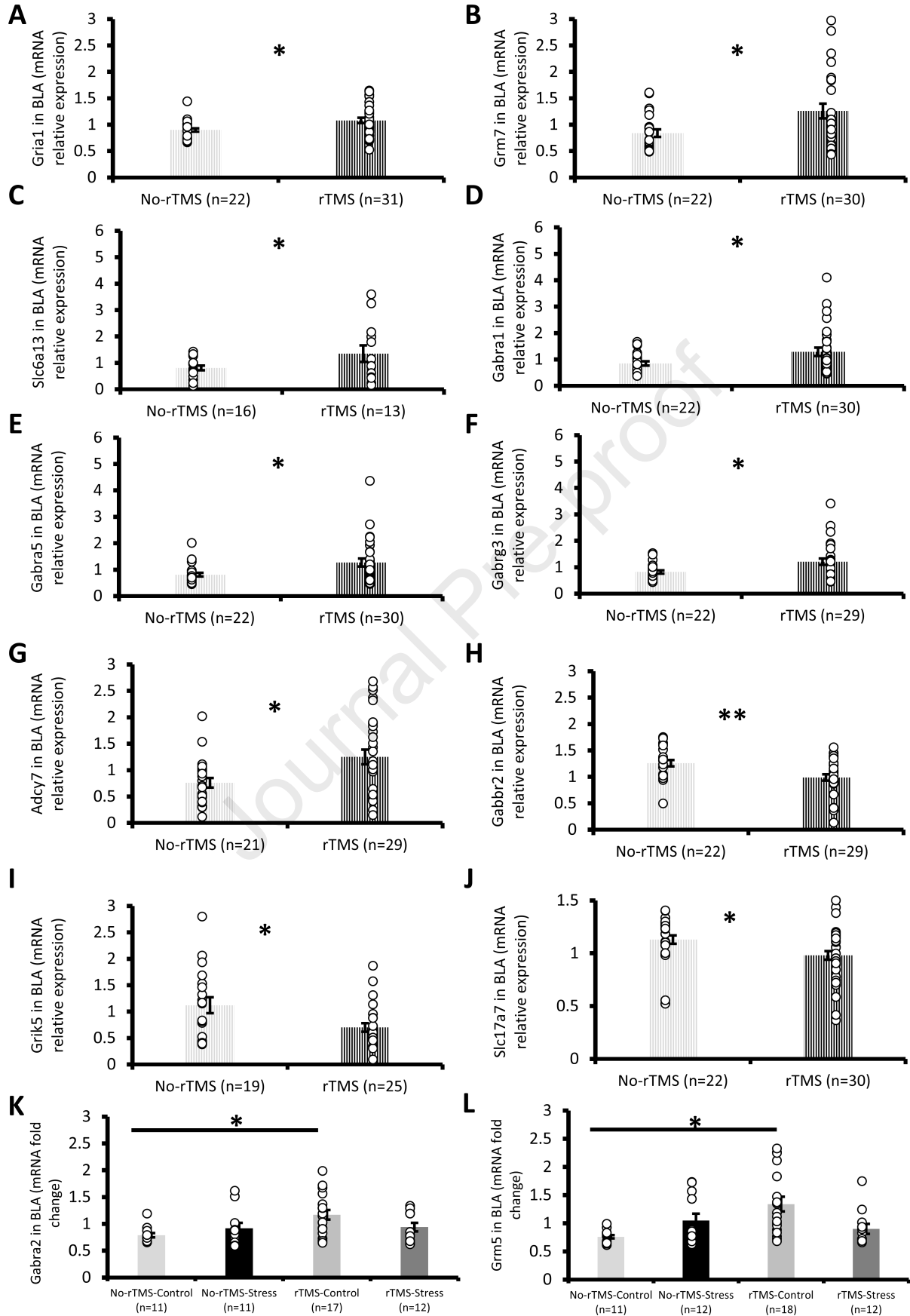
Figure 5: Results of the inflammatory pathway mRNA analysis. Differences in the IL cortex as a function of the two groups (Stress and Control), and treatment (No-rTMS or rTMS). A–B. Stress effect. A. Il1-rap. B. Tlr2. ANOVA for the stress effect: *: $p < 0.05$. C. Treatment effect. C. Nos2. ANOVA for the treatment effect: *: $p < 0.05$. D. Interaction: Il6-ra as a function of group. *: $p < 0.05$ using the *post hoc* Scheffé test. Results are expressed as mean \pm SEM.

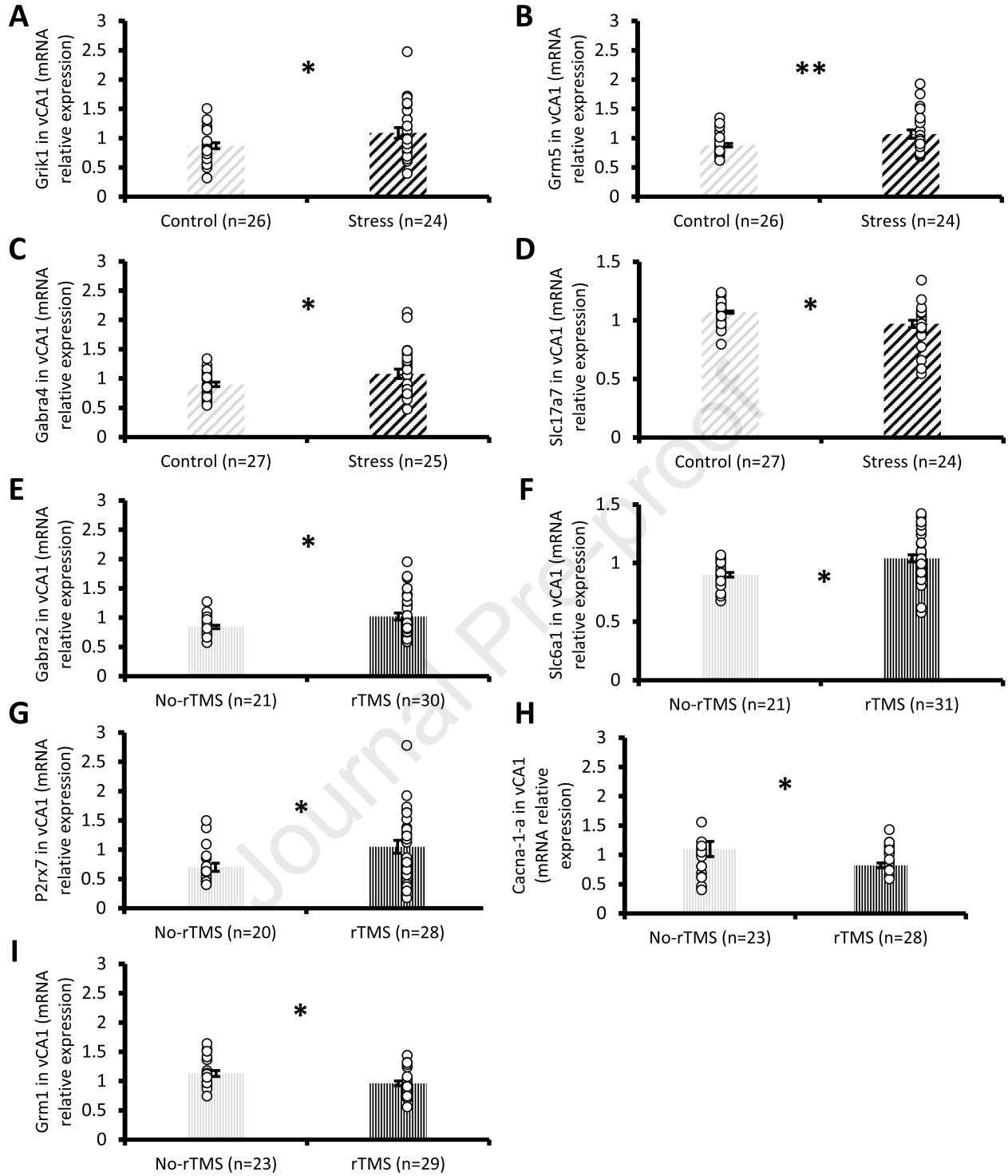
Figure 6: Results of the inflammatory pathway mRNA analysis. Differences in the BLA as a function of the two groups (Stress and Control), and treatment (No-rTMS or rTMS). A. Stress effect: Ccl11. ANOVA for the stress effect: *: $p < 0.05$. B–C. Treatment effect. B. Csf1. C. Tlr3. ANOVA for the treatment effect: *: $p < 0.05$. Interaction: Ccr3 as a function of group. *: $p < 0.05$ using the *post hoc* Scheffé test. Results are expressed as mean \pm SEM.

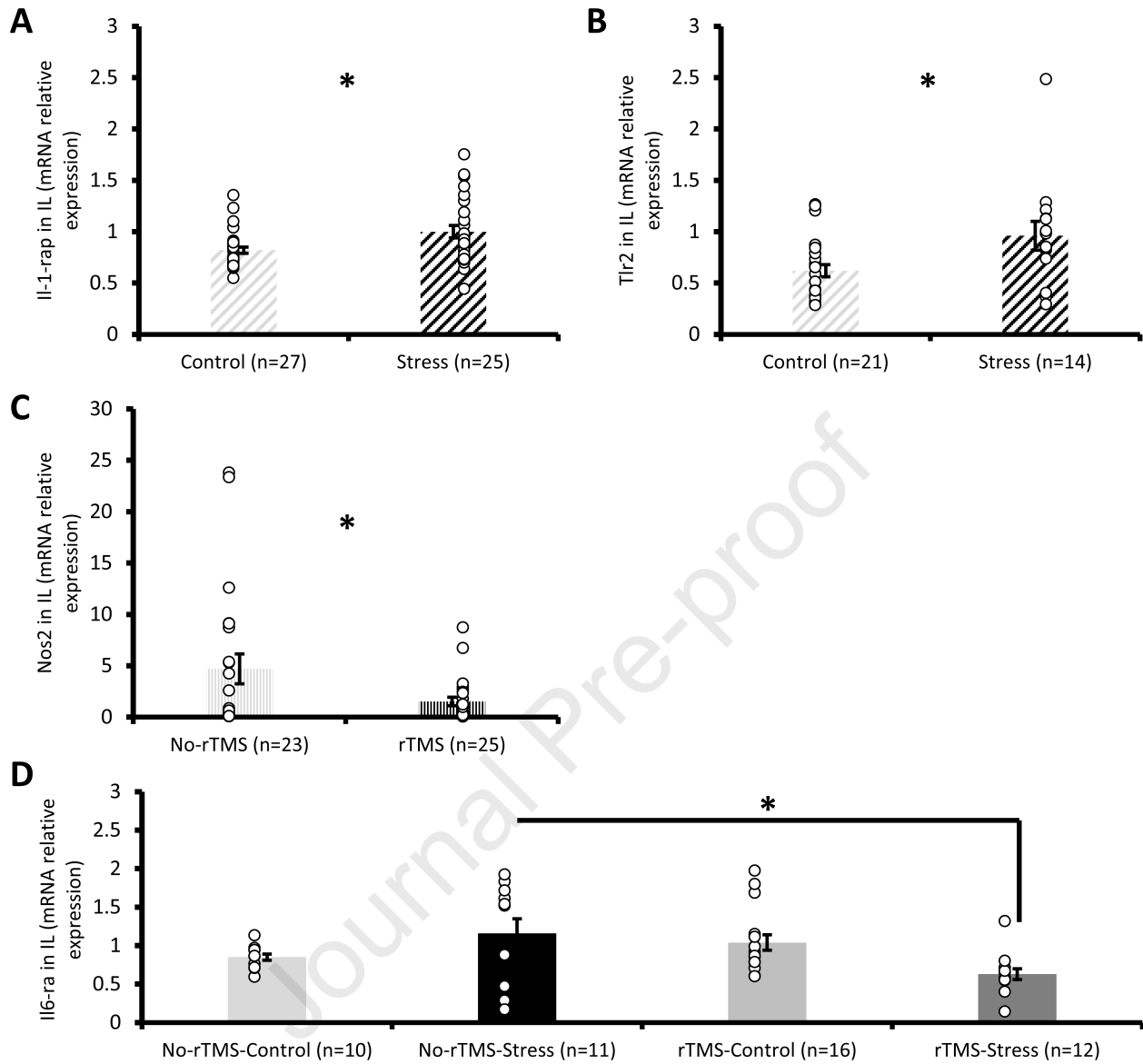
Figure 7: Results of the inflammatory pathway mRNA analysis. Differences in the vCA1 as a function of the two groups (Stress and Control), and treatment (No-rTMS or rTMS). A–C. Stress effect. A. Il6-ra. B. Fos. C. Bcl6. ANOVA for the stress effect: *: $p < 0.05$; **: $p < 0.01$. D–G. Treatment effect. D. Itgb2. E. Ltb. F. Cxcr4. G. Tlr2. ANOVA for stress and treatment effects: *: $p < 0.05$; **: $p < 0.01$. Results are expressed as mean \pm SEM.

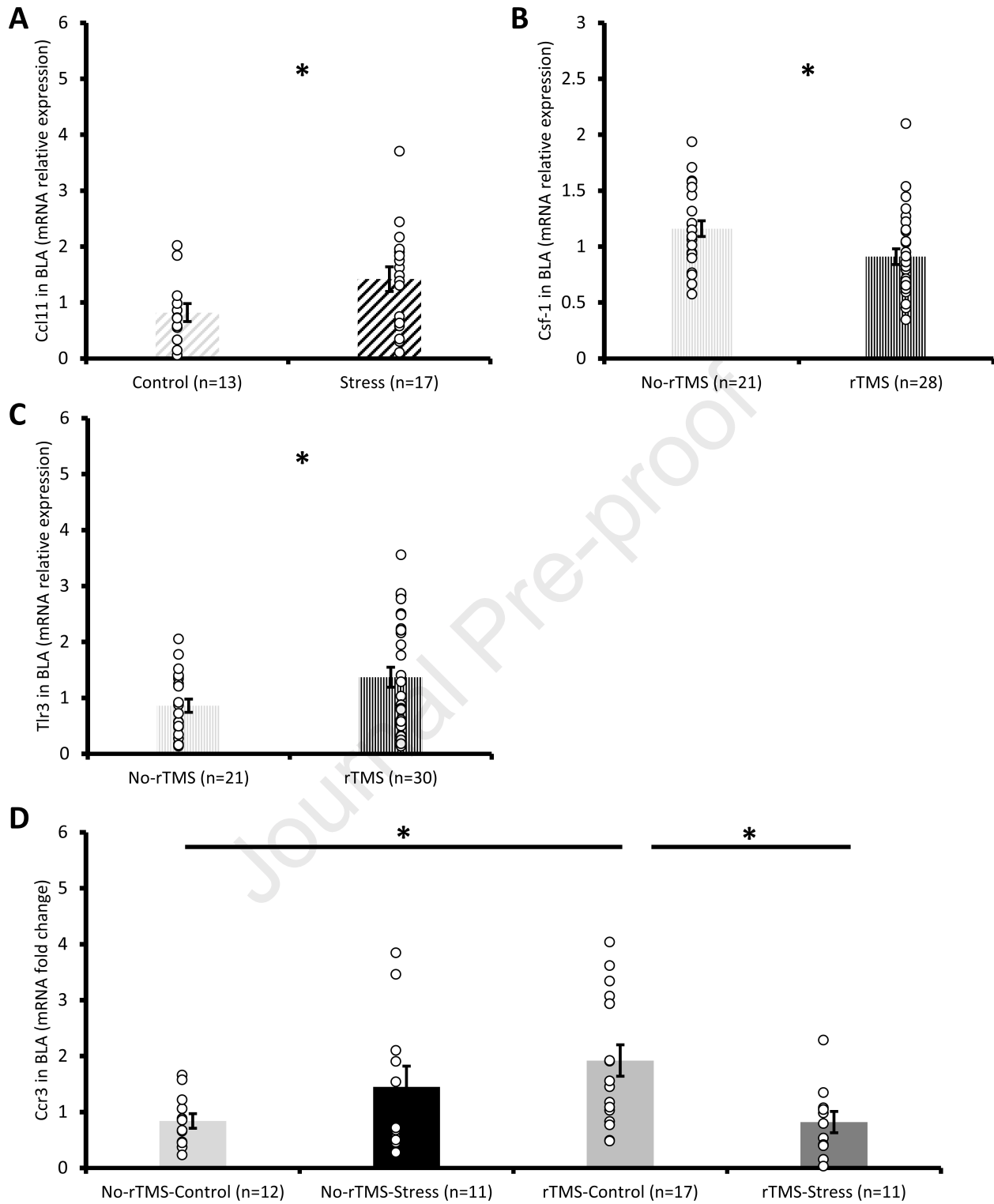


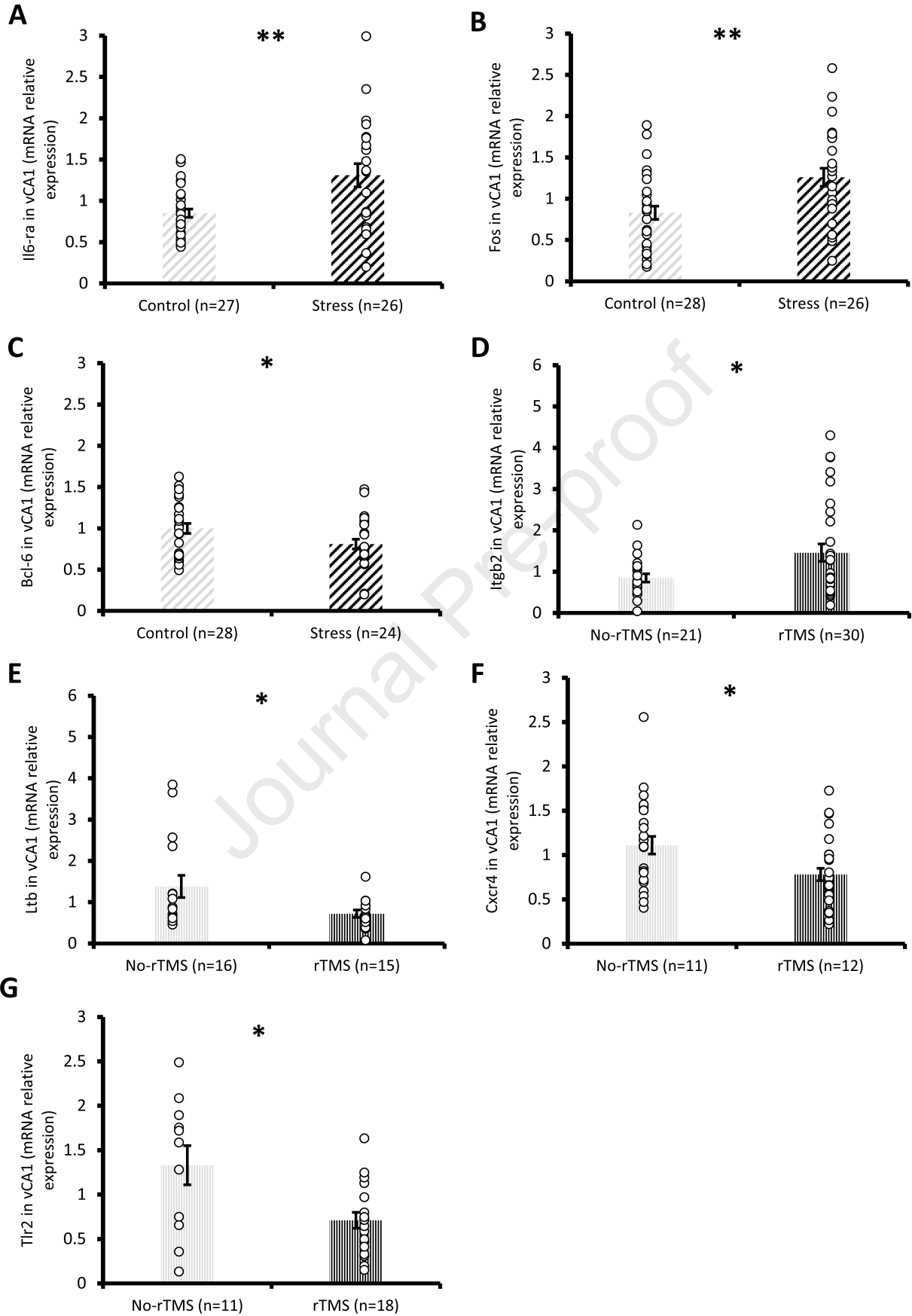












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Highlights

- rTMS focused on the prefrontal cortex decreased the fear memory phenotype.
- rTMS focused on the prefrontal cortex was associated with lower infralimbic cortex and ventral CA1 inflammation-related mRNA levels.
- rTMS focused on the prefrontal cortex was associated with higher GABA and lower glutamate neurotransmission-related mRNA in the basolateral amygdala.

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Declaration of interest

The authors declare no competing interests related to this manuscript. The opinions or assertions expressed herein are the private views of the authors and are not to be considered as official or as reflecting the views of the French Military Health Service.

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