Article

Enhanced stress response in 5-HTR overexpressing mice: altered HPA function and hippocampal long-term potentiation

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TITLE PAGE

TITLE: Enhanced stress response in 5-HT_{1A}R overexpressing mice: altered HPA function and hippocampal long-term potentiation

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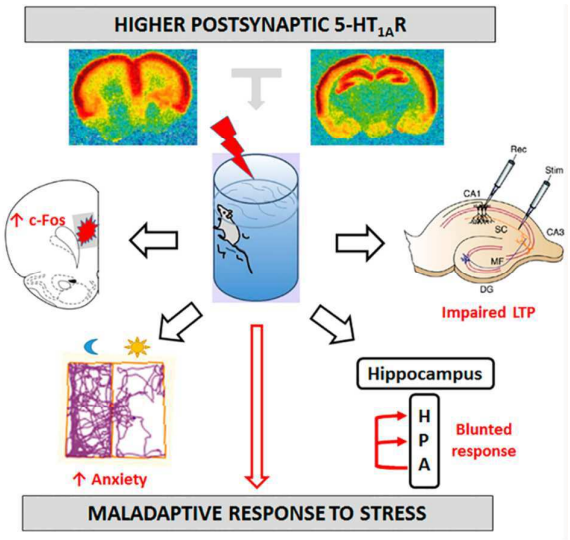
SHORT TITLE: Blunted stress response in 5-HT_{1A}R overexpressing mice

ABSTRACT

Postsynaptic 5-HT_{1A} receptors (5-HT_{1A}R) play an important role in anxiety and stress, although their contribution is still controversial. Previous studies report that mice overexpressing postsynaptic 5-HT_{1A}Rs show no changes in basal anxiety, though the influence of stress conditions has not been addressed yet. In this study, we used this animal model to evaluate the role of 5-HT_{1A}Rs in anxiety response after pre-exposure to an acute stressor. Under basal conditions, 5-HT_{1A}R overexpressing animals presented high corticosterone levels and a lower mineralocorticoid/glucocorticoid receptor ratio. After pre-exposure to a single stressor, they showed a high anxiety-like response, associated to a blunted increase in corticosterone levels and higher c-Fos activation in the prefrontal cortex. Moreover, these mice also presented a lack of downregulation of hippocampal long-term potentiation after stress exposure. Therefore, higher postsynaptic 5-HT_{1A}R activation might predispose to a high anxious phenotype and an impaired stress coping behavior.



KEYWORDS: postsynaptic 5-HT_{1A} receptor; anxiety; behavior; HPA axis; c-Fos; long-term potentiation.

INTRODUCTION

The serotonergic system is one of the key neurotransmitter systems in the body, implicated in the neurobiology of anxiety, depression and impulsive behavior, as well as in the control of other physiological functions as food intake, temperature control, sexual behavior, and the modulation of learning and memory (1). The 5-HT_{1A} receptor (5-HT_{1A}R) subtype is one of the most relevant, with a dual localization as an autoreceptor in the soma of the serotonergic neurons located in the raphe nuclei, and as a heteroreceptor in postsynaptic areas as hippocampus and cortex (2). This receptor subtype is implicated in psychiatric pathologies as bipolar disorder (3), panic disorder (4), anxiety (5), and depression (6). The involvement of the 5-HT_{1A}R in dysfunctional forms of anxiety has been studied in a wide range of preclinical research and clinical trials, including drug treatment studies, genetic research, and neuroimaging data (7). Studies in transgenic mice also demonstrate the important role of forebrain 5-HT_{1A} receptors regulating both anxious (8) and depressive (6) behaviors.

Increased circulating corticosterone levels induced by stress, have been associated to a downregulation of postsynaptic 5-HT_{1A} receptor expression and functionality in hippocampus (9, 10). Moreover, a dysregulated HPA function is associated with anxious/depressive-like behavior (11, 12). Corticosterone binds to two different receptors: mineralocorticoid receptors (MRs), present in limbic areas, and glucocorticoid receptors (GRs), more ubiquitous (13). The high-affinity MRs are saturated under basal corticosterone levels, whereas the low-affinity GRs are recruited by stress-induced high corticosteroid levels, playing a role in the negative feedback inhibition of the HPA axis (13). HPA hyperactivity leads to a downregulation of GRs and, consequently, to an impaired negative feedback inhibition (14). The subsequent glucocorticoid hypersecretion together with high serotonin release in postsynaptic areas involved in the response to stress results in the 5-HT_{1A}R downregulation in areas as the hippocampus, a neurochemical finding associated to stress-related disorders (6). In humans, a blunted cortisol variation is associated to greater scores in depressive symptoms (15).

High corticosterone levels impair long-term potentiation (LTP) in the dorsal CA1 region of the hippocampus (16), particularly, the LTP mediated by N-methyl-D-aspartate (NMDA) receptors (NMDA receptor-dependent LTP) (17). Elevated levels of corticosteroid hormones occupying both GRs and MRs (13, 18) impair the acquisition of hippocampal-dependent memories. These MRs and GRs produce opposite effects

on LTP in CA1, either facilitating or suppressing the LTP activation, respectively, leading to the induction of long-term depression (18). In fact, MR activity seems to maintain the excitability and stability of networks, while GR activation is involved in the suppression and normalization of network activity (13). Under low corticosterone levels, there is a predominant activation of MR that results in small Ca^{2+} currents that preserve the LTP (19). The exposure to high corticosterone levels promotes an additional GR activation, increasing the intracellular Ca^{2+} concentrations (20), and blocking the NMDA receptors, leading to an impaired hippocampal LTP (20).

Recently, a transgenic mouse line with a permanent 5-HT_{1A}R overexpression (OE mice) was generated (21, 22, 23). These OE mice overexpress 5-HT_{1A}R in cortex, hippocampus and other limbic areas, whereas their expression was not altered in the raphe nucleus. Previous studies using ethological- and conflict-based behavioral tests, demonstrated that the permanent postsynaptic 5-HT_{1A}R overexpression in mice does not result in significant changes in basal anxiety-related responses (22). However, we postulate that the high post/presynaptic 5-HT_{1A} receptor ratio must be influencing their vulnerability to stress. Therefore, we have evaluated the behavioral response of mice overexpressing postsynaptic 5-HT_{1A}Rs in anxiety tests following a forced swimming session used as an acute stressor. In addition, we measured the associated serum corticosterone and corticosteroid receptors levels to analyze the HPA axis functionality. c-Fos activation in the prefrontal cortex and hippocampal LTP were also evaluated in these animals under basal conditions, and following acute stress.

RESULTS AND DISCUSSION

In the present study, we demonstrate the enhanced anxiety response of mice overexpressing 5-HT_{1A}R in postsynaptic areas (5-HT_{1A} OE mice) when they are pre-exposed to a single forced swimming session used as a stressor factor, associated with an altered HPA axis response, prefrontal cortex activation and a sustained hippocampal LTP.

[^{35}S]GTPγS autoradiography: hyperfunctionality of postsynaptic 5-HT_{1A} receptors

In this study, we used a transgenic mouse line that overexpresses 5-HT_{1A} receptors in postsynaptic areas (i.e. hippocampus and cerebral cortex) (21, 23). To evaluate the 5-HT_{1A} receptor functionality, we performed a functional autoradiography using the selective 5-HT_{1A} receptor agonist 8-OH-DPAT. The agonist-mediated [^{35}S]GTPγS stimulation was significantly higher in postsynaptic areas as the medial prefrontal cortex ($p<0.01$), the CA1 area of the hippocampus ($p<0.05$) and the CA3 area of the dorsal hippocampus ($p<0.05$) in 5-HT_{1A}R OE mice. No significant differences were observed in 5-HT_{1A}R functionality in the dentate gyrus, paraventricular nucleus of the hypothalamus, and the dorsal raphe nucleus (Figure 1). Basal [^{35}S]GTPγS binding values were similar in both WT and OE animals in the different areas studied (Table S1, Supporting Information).

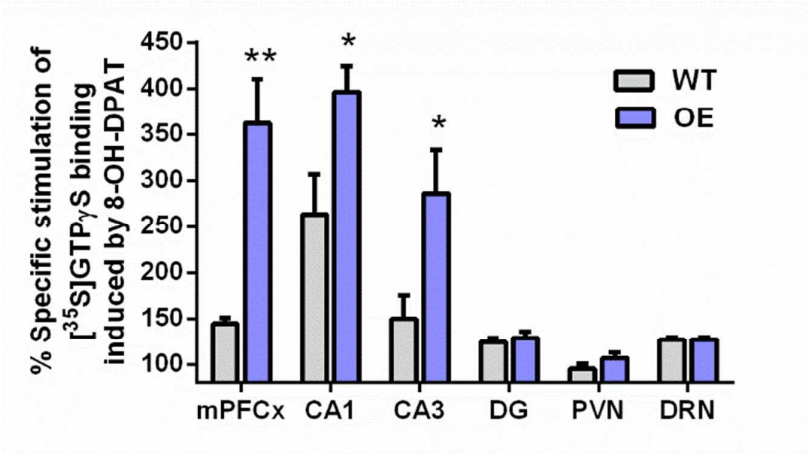


Figure 1. Autoradiographic 8-OH-DPAT-stimulated [^{35}S]GTPγS binding in WT and OE mice. Data are expressed as the mean±SEM. Student *t*-test analysis comparing OE and WT values in each brain area; *$p<0.05$ and **$p<0.01$. mPFCx (medial prefrontal cortex), CA1 (CA1 field of the hippocampus), CA3 (CA3 field of the hippocampus), DG (dentate gyrus of the hippocampus), PVN (paraventricular nucleus of the hypothalamus) and DRN (dorsal raphe nucleus). n=5-6 animals per group.

These data confirm the increased functionality of postsynaptic 5-HT_{1A}Rs overexpressed in these transgenic mice compared to their WT counterparts, with no differences in presynaptic 5-HT_{1A}Rs functionality (dorsal raphe nucleus). This higher

receptor functionality is in good agreement with the elevated receptor density observed in studies using [^{3}H]8-OH-DPAT (23). Although postsynaptic 5-HT$_{1A}$R OE mice do not present changes in 5-HT levels in projection areas compared to WT animals (22), the higher density/functionality of postsynaptic 5-HT$_{1A}$ receptors may lead to a region-dependent serotonin imbalance that could determine the behavioral outcome depending on which brain areas are engaged in different environmental challenges.

Anxiety-like responses are enhanced after acute stress in 5-HT$_{1A}$ OE mice

Taken into account the importance of the 5-HT$_{1A}$Rs in the vulnerability to stress (24, 25), we assessed the anxiety-like response of the 5-HT$_{1A}$ OE mice after the pre-exposure to an acute stressor (5 minutes forced swimming session). In the light/dark box test, non-stressed OE animals spent more time in the lit compartment than their WT counterparts ($p<0.05$). The exposure to an acute stressor immediately before behavioral testing, induced no changes in WT animals, but elicited an anxious response in OE animals, evidenced by a lower ratio light/dark time compared to the naïve OE mice ($p<0.001$) (Figure 2A). A two-way ANOVA analysis of the ratio light/dark time showed a significant effect of the stress [$F(1,31)=20.51$, $p<0.001$], and the interaction genotype x stress [$F(1,31)=18.58$, $p<0.001$]. Bonferroni posthoc analysis showed differences between WT and OE mice ($p<0.05$) and OE naïve and stressed animals ($p<0.001$), and between WT stress and OE stress mice ($p<0.05$). No changes in locomotion were observed in OE mice under basal and stress conditions (Figure S1, Supporting Information).

In the novelty suppressed feeding (NSF) non-stressed WT and 5-HT$_{1A}$R OE animals presented a similar delay to approach and eat the food pellet, whereas the pre-exposure to stress produced a higher increase in the latency to feed in OE animals ($p<0.001$) (Figure 2B). A two-way ANOVA analysis of the latency to feed showed a significant effect of the genotype [$F(1,31)=7.57$, $p<0.01$], the stress [$F(1,31)=21.99$, $p<0.001$], and the interaction genotype x stress [$F(1,31)=6.73$, $p<0.05$]. The 5-HT$_{1A}$ OE group presented lower food consumption in the post-test compared to the WT animals (Figure S2, Supporting Information), though no differences in their daily homecage consumption were detected (data not shown).

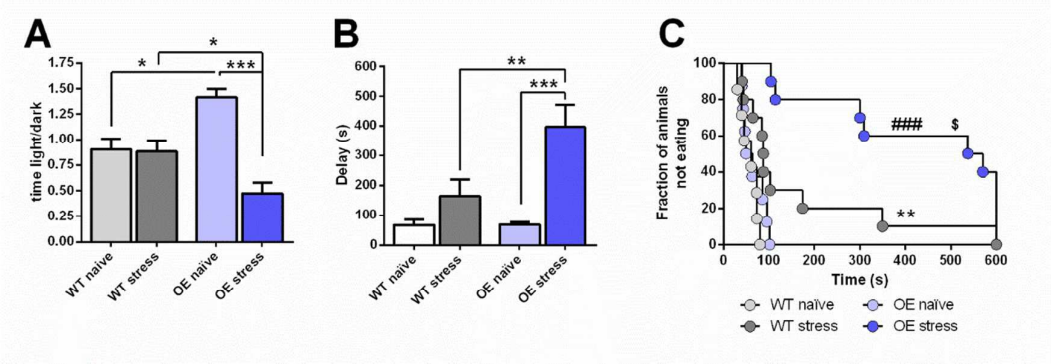


Figure 2. Anxiety- and depression-related behaviors in WT and 5-HT$_{1A}$R OE mice. (A) Ratio of time spent in the lit compartment *vs* the dark one in the light/dark box test. (B) Latency to feeding in the novelty suppressed feeding test in OE mice and WT counterparts under basal and acute stress conditions. Two-way ANOVA followed by Bonferroni post-hoc test; *p<0.05, **p<0.01, ***p<0.001. (C) Survival analysis and statistical differences between the latencies determined by the Kaplan–Meier product-limit method; **p<0.01 WT stress *vs* WT naïve, ###p<0.001 OE stress *vs* OE naïve, $p<0.05 OE stress *vs* WT stress. Note the higher anxiogenic response of OE mice when pre-exposed to stress. Data expressed as the mean±SEM. n=6-10 animals per group.

Our results demonstrate that mice overexpressing postsynaptic 5-HT$_{1A}$Rs exhibit different anxiety-like responses in non-stressed animals depending on the behavioral test. Both postsynaptic 5-HT$_{1A}$R OE mice and their WT counterparts showed a similar anxiety-like response under non-stressing conditions in the novelty suppressed feeding test, in line with previous findings in the elevated plus maze (22), though postsynaptic 5-HT$_{1A}$R OE mice displayed less anxiety-like behavior in the light/dark paradigm. It is noteworthy that postsynaptic 5-HT$_{1A}$R overexpressing mice used in this work are constitutive, which can result in compensatory changes associated with an abnormal serotonergic system functioning. In this sense, it is well known that second and third weeks of life are linked to the development of conflict-based anxiety, and that a normal 5-HT$_{1A}$ function is required to have a normal anxious phenotype (8, 25). In fact, a lower anxiety-like phenotype is observed in mice overexpressing postsynaptic 5-HT$_{1A}$ receptors during early postnatal development (21), or after postsynaptic 5-HT$_{1A}$R gain-of-function in KO animals (8). However, no changes in anxiety-like responses in no

previously stressed animals are found in 5-HT_{1A} heteroreceptor knock-out animals (25, 26).

One of the major findings of our study is the enhanced anxiety-like response (novelty suppressed feeding and light/dark box tests) in these 5-HT_{1A}R OE mice when pre-exposed to an acute stressor (forced swimming). Indeed, it is well known that the acute swim stress activates the serotonergic system (27), leading to a subsequent stress-induced increase in serotonin (28, 29). However, other factors as corticosterone play an important role in stress (30), and especially in our OE mice under acute stress conditions, as discussed below. Regarding the stress-induced increase in the latency to feed in the NSF, we cannot discard the influence of an appetite/motivational factor associated to 5-HT_{1A} receptor activation (31), since a lower drive to feed was confirmed in the post-NSF consumption test. However, the lower food consumption appears to be highly influenced by the high anxiety following the acute stressor elicited by these 5-HT_{1A} OE mice, since the food consumed daily in their homecage was similar to their WT counterparts.

Blunted HPA in OE mice in response to stress

Bearing in mind the increased anxiety-like response elicited in acutely stressed 5-HT_{1A}R OE mice, we decided to check the status of the hypothalamic-pituitary-adrenal (HPA) axis. In non-stressed animals, corticosterone levels were higher in OE than in WT mice ($p<0.01$). The exposure to acute stress induced an increase in serum corticosterone levels in WT animals ($p<0.001$), but no changes in OE animals (Figure 3A). A two-way ANOVA analysis of serum corticosterone levels showed a significant effect of the stress [$F(1,33)=7.65$, $p<0.01$], and the interaction genotype x stress [$F(1,33)=15.95$, $p<0.001$]. Moreover, the effects of the circadian rhythm on the corticosterone levels differed between both groups, leading to a lower corticosterone peak in 5-HT_{1A} OE animals ($p<0.001$) (Figure S3, Supporting Information).

We also evaluated the mRNA expression of MR and GR using qPCR (Figure 3B). MR mRNA expression was lower in OE mice compared to their WT counterparts (two-tailed t-test, $t14=2.49$; $p<0.05$), while GR mRNA expression was significantly higher in OE mice compared to WT animals (two-tailed t-test, $t14=2.22$; $p<0.05$). The ratio MR/GR expression was significantly lower in OE mice compared to WT animals (two-tailed t-test, $t14=4.37$; $p<0.001$).

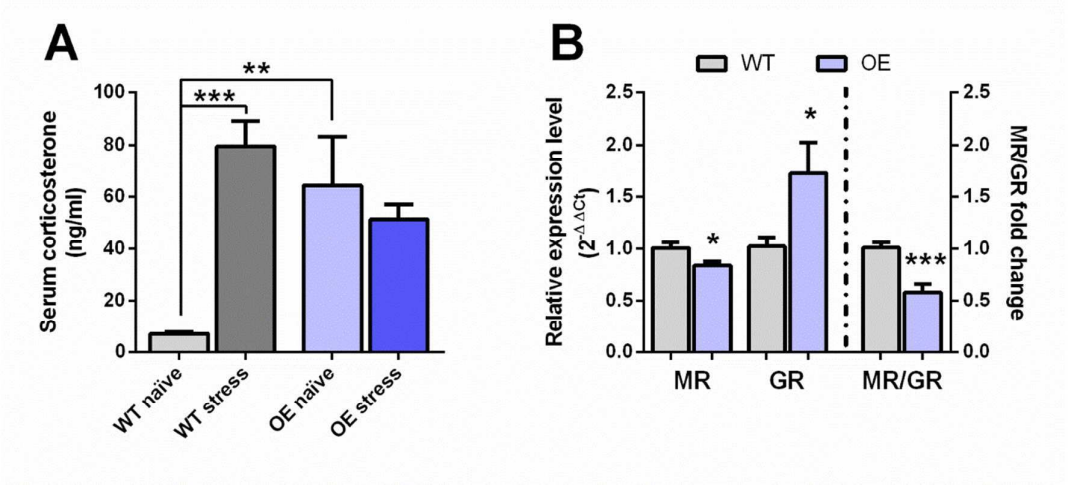


Figure 3. HPA response to stress in WT and OE 5-HT_{1A}R mice. A) Serum corticosterone levels in WT and 5-HT_{1A}R OE mice under basal conditions and after acute stress. Serum corticosterone levels are expressed as ng/ml. Data are expressed as the mean±SEM. Two-way ANOVA test followed by Bonferroni post-hoc test; **p<0.01 and ***p<0.001. n=7-9 animals per group. B) Corticosteroid mRNA receptor expression in the hippocampus of WT and 5-HT_{1A}R OE mice; MR expression, GR expression and MR/GR expression ratio. Data are expressed as the mean±SEM. Student's t-test, two-tailed; *p<0.05, ***p<0.001. n=6-8 animals per group.

The enhanced anxious response to acute stress in postsynaptic 5-HT_{1A}R OE mice is associated with elevated basal corticosterone, without modifications of corticosterone levels following stress (blunted HPA axis response) compared to their WT counterparts, in line with previous findings (30). These elevated basal corticosterone levels are in good agreement with that observed in animal models of stress (9, 32). Furthermore, our 5-HT_{1A}R OE mice present a lower diurnal variation of corticosterone levels (32). This reduced corticosterone peak in 5-HT_{1A} OE mice parallels the findings reported in patients suffering from post-traumatic stress disorder (33, 34). Postsynaptic 5-HT_{1A}Rs, especially those localized in the paraventricular nucleus, are involved in stress regulation since their activation leads to increased corticosterone levels (35). The 5-HT_{1A}R density is increased in the hypothalamus of these animals (23). However, we were not able to detect a significant change in the 5-HT_{1A}R functionality probably

due to the high basal [^{35}S]GTPγS binding levels in this area. Therefore, it is difficult to draw a conclusion about the role of 5-HT$_{1A}$ hypothalamic receptors in the modulation of corticosterone levels.

In the postsynaptic 5-HT$_{1A}$R overexpressing mice, high basal corticosterone levels are associated with lower levels of MR mRNA expression in the hippocampus, in good accordance with previous studies (9, 10). The lower MR/GR mRNA expression ratio observed in OE animals is also observed in animal models of stress (36), and contrast with the higher ratio in animals with low-anxiety phenotype (37). This unbalanced corticoid receptor expression is also present in rats with low 5-HT$_{1A}$R expression in DRN (38), mirroring the high post/presynaptic 5-HT$_{1A}$R ratio present in our animals. Interestingly, mice overexpressing MR in forebrain areas show an opposite behavior with a less anxious phenotype induced by subsequent behavioral exposures (37). Postsynaptic 5-HT$_{1A}$R OE mice also presented high levels of GR mRNA expression similarly to animals with a high anxiety profile (39, 40), and a blunted HPA response (40). It could be speculated that the high GR mRNA expression in OE animals may result in a hyperactive negative feedback, and the subsequent impaired HPA response following acute stress (41), though it requires further investigation (i.e.: dexamethasone suppression test). A blunted response to stress has been reported after corticosterone infusion in mPFCx (42), an area associated with stress habituation, suggesting that the high basal corticosterone levels diminish the HPA stress response. Moreover, increased activity in mPFCx, induced by the local administration of picrotoxin, is associated with a lower stress-induced corticosterone secretion (43).

c-Fos activation in mPFCx induced by stress

Taking in consideration the role of the mPFCx in stress-related responses, we next analyzed the neural activation level by means of c-Fos immunohistochemistry. Our results demonstrate that the c-Fos expression in mPFCx was increased in OE stressed animals ($p<0.001$), while no changes were observed in the WT animals (Figure 4). A two-way ANOVA analysis of the c-Fos expression showed a significant effect of the genotype [$F(1,21)=8.90$, $p<0.01$], the stress [$F(1,21)=39.39$, $p<0.001$], and the interaction genotype x stress [$F(1,21)=11.33$, $p<0.01$].

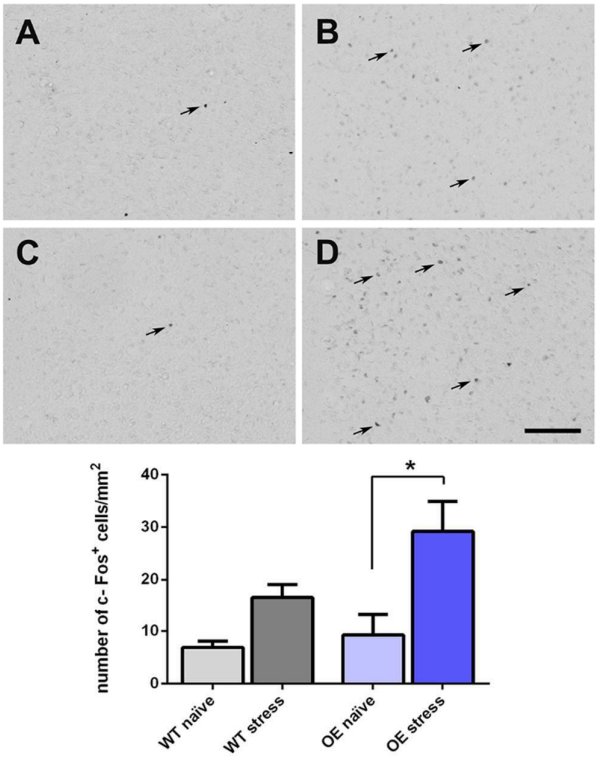


Figure 4. Activation of c-Fos in the medial prefrontal cortex of the WT and 5-HT_{1A} OE mice under basal conditions and after acute stress. Two-way ANOVA followed by Bonferroni post-hoc test; *p<0.05. Data expressed as the mean±SEM. n=5-6 animals per group. Representative microphotographs are shown for c-Fos expression in: A) WT naïve, B) WT stress, C) OE naïve, and D) OE stress. c-Fos positive cells are marked with arrows. Bar: 100 μm.

In our study, the higher mPFCx c-Fos activation after stress may account for the absence of corticosterone secretion boost following stress (43). Moreover, the prefrontal cortex is involved in the regulation of stress as an area integrating cognitive/affective information with HPA axis functioning (44, 45). Indeed, activation of the prefrontal cortex, measured by increased c-Fos expression, is reported following stress-inducing procedures (i.e.: FST, immobilization) (46, 47, 48), in line with our findings. However, other authors indicate that the 5-HT_{1A}R-mediated inhibition of c-Fos expression in mPFCx is associated with a higher anxiety (49). It has been reported that high anxious phenotype is associated to predominant 5-HT_{1A} activation on GABA interneurons, leading to an increased pyramidal hyperactivity (50, 51). In good

agreement, studies in GAD65 knock-out animals indicate increased fear and anxiety behavior similar to models of posttraumatic stress disorder (52). Further experiments are needed to clarify whether the overexpression of postsynaptic 5-HT_{1A}R OE in these mice leads to disturbances in the tone of GABAergic inhibition, and consequently, increased pyramidal neuron activation, responsible for a higher level of anxiety.

Lack of long-term potentiation (LTP) impairment in OE mice after acute stress

Finally, taking into consideration the importance of LTP in stress response, we studied the effect of stress on dorsal hippocampal CA1 LTP. To assess the effect of 5-HT_{1A}R overexpression on the hippocampal synaptic transmission and plasticity, we recorded fEPSPs from hippocampal slices of WT and OE mice in non-stressed animals, and after acute exposure to a stressing event. A theta burst stimulus was used to induce LTP at the Schaffer collateral-CA1 pathway. No significant differences were found in fEPSP amplitudes in the baseline of WT and OE mice. Comparison of LTP induction in hippocampal slices of non-stressed WT and OE animals showed no differences in the total LTP between both genotypes. As expected, LTP impairment was observed in stressed WT mice ($p<0.01$) (Figure 5A). By contrast, LTP measured in hippocampal slices of stressed OE mice did not show significant differences compared to those not subjected to a stressor (Figure 5B).

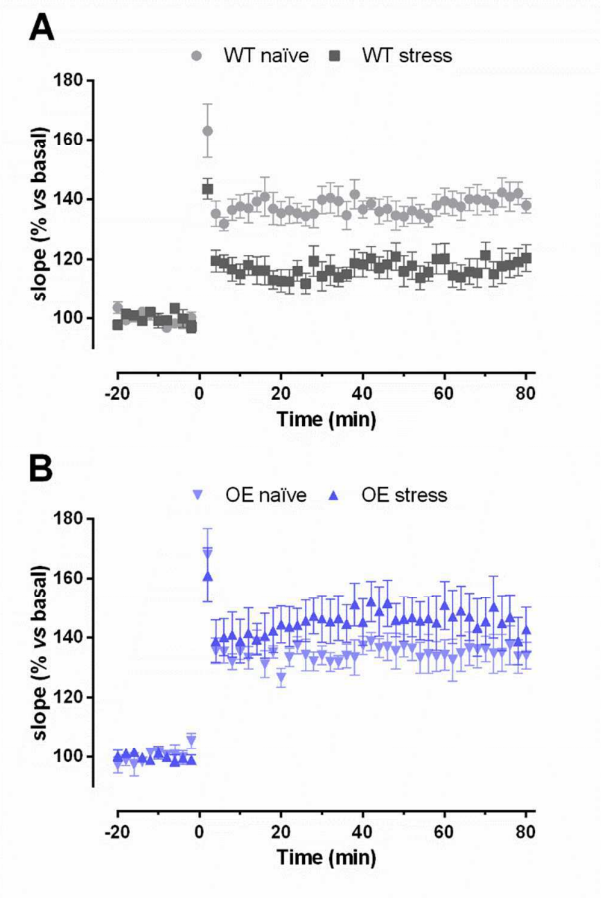


Figure 5. Lack of changes in hippocampal LTP after acute stress in 5-HT_{1A}R OE mice. Time courses of the initial slope of fEPSPs recorded from the apical dendritic layer of the CA1 region in hippocampal slices after stimulation of the Schaffer collateral-commissural pathway at 30 s intervals. After 20 min of stable baseline recording, a theta burst stimulus induced a robust and similar LTP in hippocampal slices of WT and 5-HT_{1A}R OE mice. The stress resulted in the significant down-regulation of the LTP in WT animals ($p<0.01$) (A), but not in OE mice (B). Data are presented as mean±SEM from one slice per animal n=5-7 per group.

These results demonstrate that the higher density and functionality of 5-HT_{1A}Rs in the dorsal hippocampus of OE mice does not affect LTP in basal conditions. Acute stress exposure in WT animals induces a downregulation on the LTP in dorsal hippocampus, as previously reported (16, 17, 53, 54), while does not induce changes in 5-HT_{1A}R OE mice. The LTP downregulation in dorsal hippocampus is associated to increased

corticosterone levels (16, 54) after uncontrollable stress (54), acting through GR receptors (55). Thus, our results strongly suggest that the absence of a stress-induced increase in corticosterone levels in 5-HT_{1A} OE mice is underlying the lack of LTP downregulation following an acute stressing event in these animals.

Overall, mice overexpressing 5-HT_{1A}R in postsynaptic areas showed exacerbated anxious response to acute stress, a behavioral outcome associated with the inability to modulate the HPA axis in response to stressors, increased prefrontal cortex activation, and the lack of hippocampal LTP downregulation. A similar impaired stress response is present in mice with high anxiety-related behavior and in some pathologies as in post-traumatic stress disorder (PSTD) (56), and depression (40, 57). Therefore, a higher 5-HT_{1A}R post/presynaptic ratio results in an impaired stress coping behavior that may increase the susceptibility to develop stress-related emotional disorders.

METHODS

Animals

Permanent postsynaptic 5-HT_{1A}-receptor overexpressing (OE) mice strain and their wild-type (WT) counterparts were generated as previously indicated (21, 22). These mice strain was created on an out-bred NMRI genetic background. They exhibit a higher 5-HT_{1A} receptor density in hippocampus, cortex, amygdala, and hypothalamus, areas in which these receptors are located at the postsynaptic level. However, no changes are found in the density of 5-HT_{1A} autoreceptors in the raphe nuclei (22, 23). Male mice (10-14 weeks) were grouped housed (n=4-5) under controlled conditions (22±1ºC; 12 h light/dark cycle) with food and water ad libitum. All procedures were carried out with the previous approval of the Animal Care Committee of the University of Cantabria and according to the Spanish legislation (RD 53/2013) and the European Communities Council Directive (2010/63/UE) on “Protection of Animals Used in Experimental and Other Scientific Purposes”.

Two different types of studies were designed. First, different sets of WT and OE animals were used in autoradiography (n=5-6 per group) and qPCR assays (n=6-8 per group). Second, four experimental groups (WT naïve, WT stress, OE naïve, and OE

stress mice) were used to assess the effect of acute stress in the following experimental procedures: behavioral testing (n=6-10 per group), serum corticosterone measurement (n=5-9 per group), c-Fos immunohistochemistry (n=5-6 per group), and electrophysiology studies (n=5-7 per group). A different set of animals was used for each experimental technique.

Acute stress protocol: a single forced swimming session was used as acute stressor. Mice were placed in swimming tanks 12 cm in diameter and 24 cm tall. The tank was filled with enough water at 25-27°C so that the mice could not touch the bottom. Each mouse was placed individually in the swimming tank for a single 5 min session, gently dried up using a paper towel, and immediately used in the different experimental procedures.

Experimental design for "stress" studies (see chronogram in Figure 6): naïve and stressed animals were subjected to a battery of behavioral and neurochemical analyses. A) light/dark box and novelty suppressed feeding tests, with a one-week delay between both; B) blood extraction for serum corticosterone determination; C) animal perfusion 2 hours after the stressor for c-Fos immunohistochemistry studies; and D) *in vivo* recording of long-term potentiation in hippocampal slices.

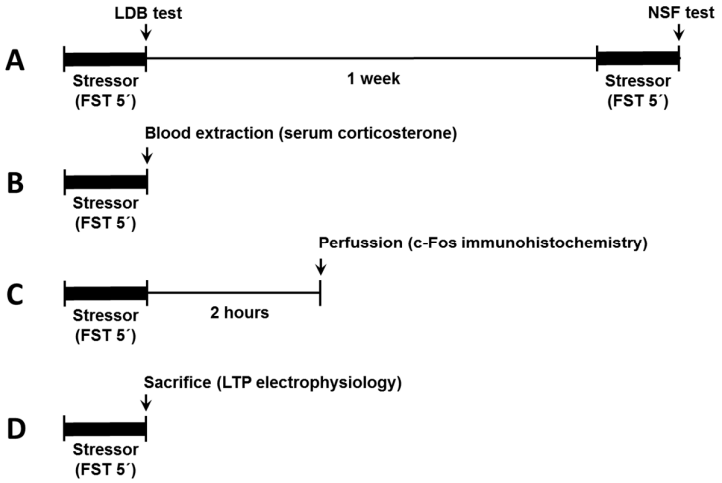


Figure 6. Experimental timeline for the acute stressing procedure (5 min forced swimming session), in the stressed groups. A) Light/dark box (LDB) and novelty suppressed feeding (NSF) tests; B) blood sampling immediately after the acute stress; C) perfusion 2 h after the stressing event in the c-Fos immunohistochemical assays;

and D) hippocampal sectioning immediately after the 5 min swim session for the long term potentiation experiments.

Autoradiography for 5-HT_{1A} receptor function

Experiments were performed following a previously described protocol (58). Sections were pre-incubated for 30 min at 25°C in a buffer containing 50 mM Tris-HCl, 0.2 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT and 2 mM GDP (pH 7.7), and subsequently incubated for 2 h at 25°C in the same buffer containing 3 mU/ml adenosine deaminase and 0.04 nM [^{35}S]guanosine-5-O-(3-thio) triphosphate (GTPγS; PerkinElmer Inc., Waltham, MA, USA). Consecutive sections were incubated with 10 μM of the selective 5-HT_{1A}R agonist (±)-8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT; Sigma-Aldrich, Spain) alone or in the presence of 10 μM of the selective 5-HT_{1A}R antagonist WAY100635. Non-specific binding was determined in the presence of 10 μM GTPγS. After the incubation, the sections were washed twice for 15 min in 50 mM Tris-HCl buffer (pH 7.4) at 4°C, rinsed in distilled cold water and cold air dried. Sections were exposed to radiation-sensitive films (BioMax® MR film, Sigma-Aldrich, San Luis, MI, USA) together with ^{14}C-polymer standards (Amersham, UK) for 2 days at 4°C. 8-OH-DPAT stimulated values are represented as percentage versus basal (100%).

Behavioral tests

Light/dark box test: The light/dark box test was performed in an arena formed by two equally sized compartments (15 cm wide × 30 cm long × 20 cm high) separated by an opening located centrally at floor level (6 cm wide x 6 cm high) (59). A dark compartment had black walls covered with a lid. The other compartment (not covered) had see-through walls and was lit by a light bulb (400 lux). Mice were placed in one corner of the light compartment. The latency of the first entry, time spent, number of entries into the brightly lit compartment, and total distance traveled in both compartments, were recorded during 5 min testing session using Any-maze Video-Tracking software (Stoelting Co., Wood Dale, IL, USA). The anxiety level was represented as the ratio of the time spent in the light vs the dark compartment.

Novelty suppressed feeding: This test was performed 7 days after the light/dark box test. It was performed following a previous protocol (60) with minor modifications. Mice were placed into an open field arena (50x50x30 cm; luminance 40-50 lux), from the corner after 24 h food deprivation. The first latency to eat a single food pellet placed in the center during a 10 min session was recorded (defined as the mouse biting the food) using the Any-maze Video-Tracking software (Stoelting Co.). Immediately after this test, the animal was transferred to its home cage, and the food consumption for each mouse was measured during a 5 min period. The animals that did not eat during the test (but ate in the post-test), were assigned a value of 600 s. The animals that did not eat during the post-test were discarded from the results analysis.

c-Fos immunohistochemistry

Mice were anesthetized with an injection of sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in PBS. Brains were post-fixed and cryoprotected with 30% sucrose. Serial coronal sections (40 μm) of the brains were obtained. Sections were treated with 0.3% H_2O_2 in phosphate-buffered saline (PBS) for 10 min and blocked with 0.2% Triton X-100 and 2% normal donkey serum at room temperature for 1 h. Sections were incubated with rabbit anti c-Fos primary antibody (1:1000; Santa Cruz Biotechnology) in PBS containing 3% normal donkey serum, at 4ºC overnight. Sections were washed in PBS-T, and incubated with a biotinylated donkey anti-rabbit IgG secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and amplified with avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA). c-Fos positive cells were labeled using diaminobenzidine (DAB) + Ni as chromogen (Vector Laboratories).

c-Fos-containing cells were counted in the medial prefrontal cortex including prelimbic and infralimbic regions (from 1.98 to 1.70 mm relative to Bregma) (61). Nuclei counting was performed by a blind observer to experimental groups. Data are presented as c-Fos positive cells per area (mm^2).

Microscope image acquisition

c-Fos immunohistochemistry was visualized using a brightfield microscope Zeiss Axio Scope.A1, using a 10x magnification, and 0.25 numerical aperture of the objective lenses. The image acquisition was performed using a Zeiss AxioCam HRc camera, and

the acquisition software used was Micro-Manager (version 1.4.22) (62). The images were processed using an Adobe Photoshop CC software (version 14.0) (Adobe Systems Software Ireland Ltd., Ireland).

Serum corticosterone levels measurement

Blood samples were collected between 9 and 11 a.m. in the different experimental groups (WT and OE, stressed and non-stressed). For the corticosterone variation during the day, blood samples were collected every 4 hours in WT and OE non-stressed animal groups, starting at 10 a.m. Mice were deeply anesthetized with sodium pentobarbital (40 mg/kg, i.p.) to avoid procedure stress. The mice tails were cut and whole blood was collected in tubes (100 µl volume approximately). The blood was led to clotting during 20-30 minutes and centrifuged twice at 2000 xg for 10 minutes to completely remove the debris. The serum was stored at -20ºC until use. The corticosterone amount present in the serum samples was measured using a corticosterone ELISA Kit (Abcam plc, UK) as indicated by the supplier. Briefly, serum samples were diluted 1:5 and incubated together with biotinylated corticosterone in the plate wells provided in the kit during 2 hours at room temperature. Then, the wells were washed and added streptavidin-peroxidase conjugate and incubated for 30 min. After that, the plate was washed and incubated for 30 min with the chromogen substrate. The reaction was stopped and the absorbance was measured at 450 nm. The standard curve data were fitted to a four-parameter logistic nonlinear regression curve.

Quantitative PCR

Mice were killed by decapitation, the brains rapidly removed from the skulls, and the hippocampi dissected, immediately frozen and stored at -80ºC until used. Total RNA and whole protein homogenate were extracted using Tripure™ Isolation Reagent (Sigma-Aldrich) according to the manufacturer's instructions.

Once purified, the quality and concentration of the sample were tested measuring the absorbance (260/280 nm) with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA was reverse transcribed to cDNA using the High Capacity

cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative polymerase chain reaction (qPCR) was performed in triplicate using gene-specific primers and SYBR® Green on an ABI PRISM 7500 real-time thermal cycler (Applied Biosystems). Oligonucleotide primers were designed using Primer Express software (Applied Biosystems). The primer sequences and the GenBank accession numbers are given in Table 1.

Table 1. Primers used in qPCR studies and GenBank accession numbers.

Gene	Forward	Reverse	NCBI reference
MR (Nr3c2)	5'-GGCTACCACAGTCTCCCTGA-3'	5'-ACGTTGACAATCTCCATGTAG-3'	NM_001083906.1
GR (Nr3c1)	5'-TGGGACTGTATATGGGAGAG-3'	5'-GGTTTGCAATGCTTTCTTCC-3'	NM_008173.3
GAPDH	5'-ACAGTCCATGCCATCACTGCC-3'	5'-GCCTGCTTCACCACCTTCTTG-3'	NM_008084
18S (Rn18s)	5'-CTTAGAGGGACAAGTGGCG-3'	5'-ACGCTGAGCCAGTCAGTGTA-3'	NR_003278
Actb	5'-CTCTGGCTCCTAGCACCATGAAGA-3'	5'-GTAAAACGCAGCTCAGTAACAGTCCG-3'	NM_007393

Relative changes in the expression of the target genes were determined using the following equation: fold change=$2^{-\Delta\Delta Ct}$, where ΔCt=(Ct target – Ct 18S rRNA) and ΔΔCt=ΔCt (for the experimental condition) – ΔCt (for the control condition) (63). These experiments were carried out according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (64).

Long-term potentiation (LTP)

Mice were decapitated and the brains were rapidly removed. The hippocampi were dissected and 400 μm slices were cut with a tissue chopper (65). Slices were allowed to recover for at least 1 h in an interface chamber at room temperature with artificial CSF containing the following: 120 mM NaCl, 3.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM D-glucose (saturated with 95% O_2 and 5% CO_2). Field EPSPs (fEPSPs) were recorded from the CA1 stratum

radiatum of dorsal hippocampus with a glass micropipette (1–4 MΩ) containing 2 M NaCl and evoked by stimulation of the Schaffer collaterals with insulated bipolar platinum/iridium electrodes >500 μm away from the recording electrode. The stimulus strength was adjusted to evoke fEPSPs equal to 50% of the relative maximum amplitude without superimposed population spike. After stable baseline recordings (100 μs pulse duration, 0.033 Hz), LTP was induced by theta burst stimulation (10 trains of five pulses at 100 Hz and intervals of 200 ms). The duration of the stimulation pulses was doubled during tetanus. fEPSPs were amplified, bandpass filtered (1 Hz to 1 kHz), and stored in a computer using the Spike 2 program (Cambridge Electronic Design, Cambridge, UK). For the analysis, fEPSP slopes were expressed as a percentage of the baseline values recorded.

Data analysis

Results are shown as mean ± standard error of the mean. The statistical analysis of the results was performed using Student's t-test or two-way ANOVA (genotype and stress as main factors) followed by Student-Newman-Keuls posthoc test. The statistical analysis in the electrophysiological experiments was performed using repeated-measures (RM) multivariate ANOVA (MANOVA) (time x stress x genotype). The statistical analysis test and the number of animals used for each experimental set are indicated in the results section and figure legends. The level of significance was set at $p<0.05$. Graphs and the statistical analyses were calculated using the GraphPad Prism 5.01 software (GraphPad Software, GraphPad, USA), or SPSS for Windows version 18.0.

SUPPORTING INFORMATION

Table S1. Absolute values (nCi/g tissue) of basal [^{35}S]GTPγS binding.

Figure S1. Total distance traveled in the light/dark box test.

Figure S2. Food eaten in the novelty suppressed feeding post-test.

ABBREVIATIONS:

5-HT_{1A} receptor (5-HT_{1A}R) overexpressing (OE) mice, forced swimming test (FST), open field (OF), novelty suppressed feeding (NSF) test, mineralocorticoid receptor (MR), glucocorticoid receptor (GR), long-term potentiation (LTP).

AUTHOR INFORMATION

Author contributions:

FP-C designed and performed experiments, analyzed and interpreted data, and drafted the manuscript. RV performed electrophysiological experiments, analyzed and interpreted data. AD designed experiments, analyzed and interpreted data, and drafted the manuscript. EG-M performed immunohistochemistry studies. RL performed the corticosterone measurement and quantitative PCR experiments. EC designed experiments, analyzed and interpreted data, and drafted the manuscript. RH performed the behavioral studies. HF performed the critical revision of the manuscript. BB analyzed and interpreted the data, and drafted the manuscript. JB analyzed and interpreted the data, and drafted the manuscript. BR performed the corticosterone measurement. BC-F performed the critical revision of the manuscript. AP designed experiments and performed the critical revision of the manuscript.

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Conflict of interest:

The authors declare no competing financial interests.

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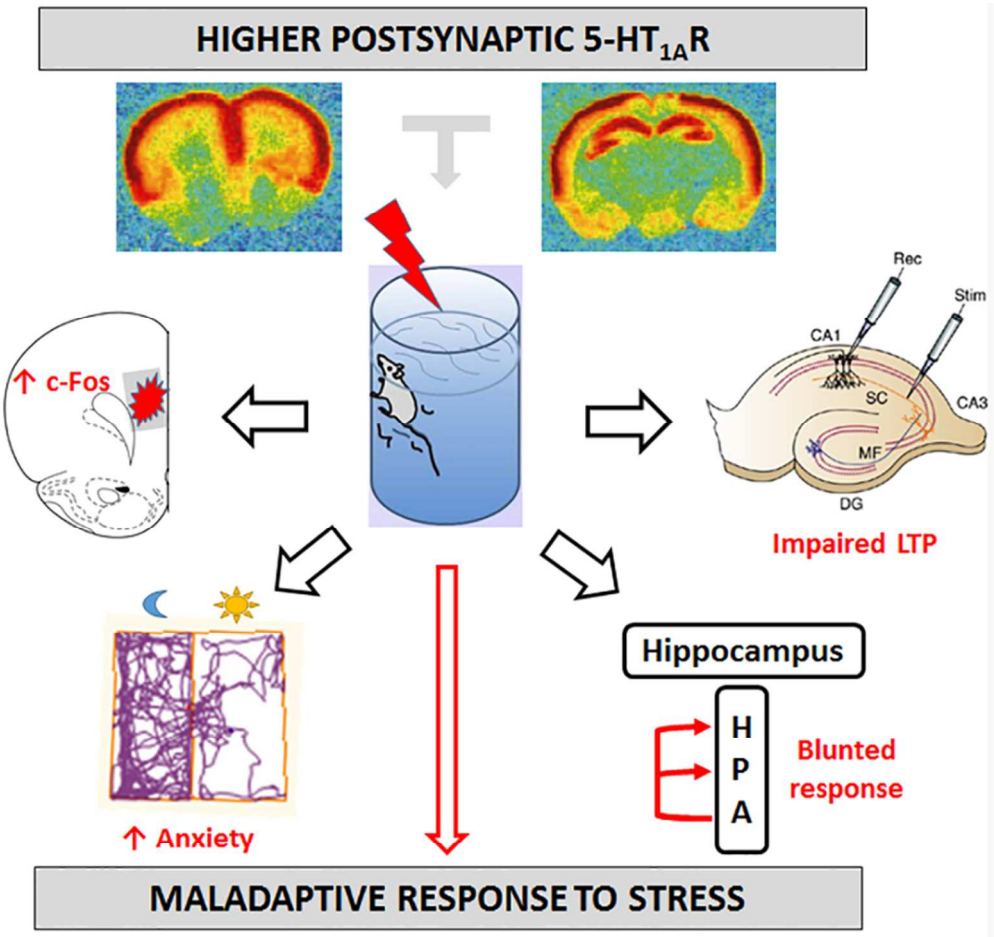

Graphical table of contents

80x75mm (300 x 300 DPI)

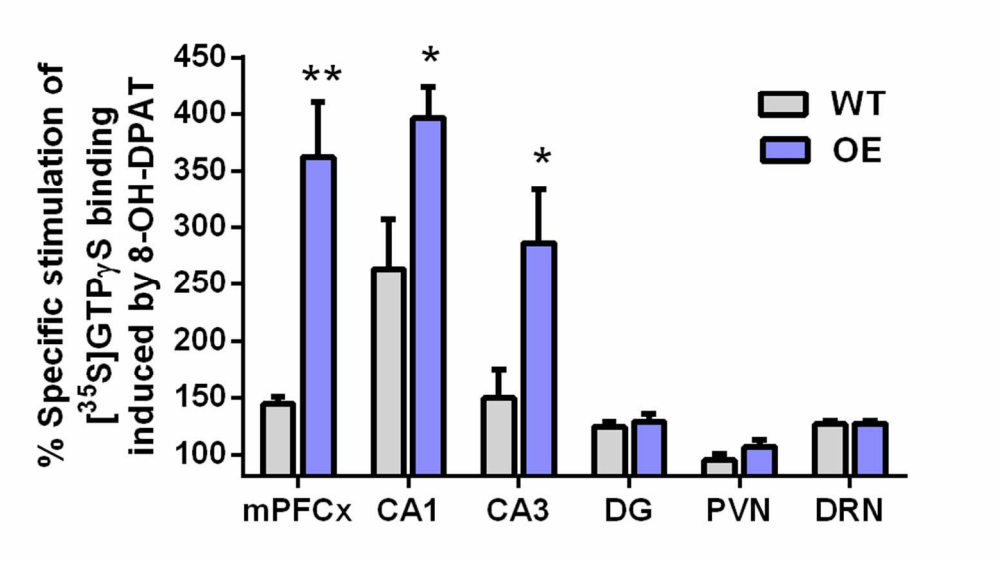

Figure 1

85x48mm (300 x 300 DPI)

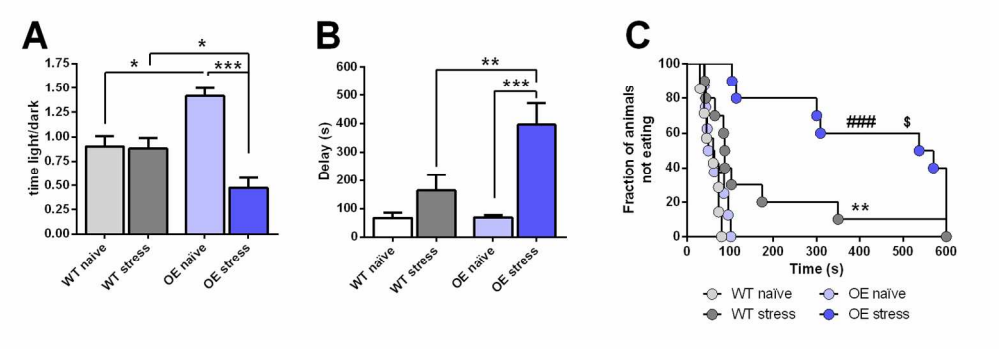

Figure 2

140x49mm (300 x 300 DPI)

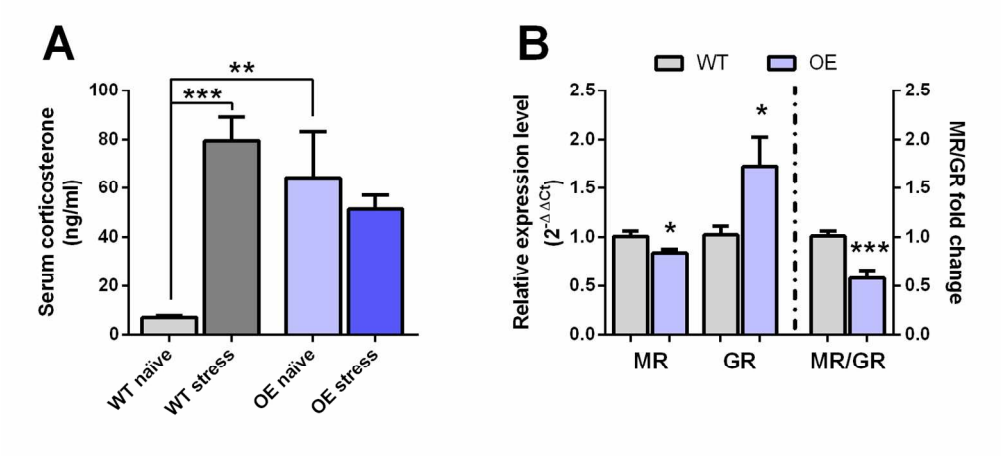

Figure 3

140x64mm (300 x 300 DPI)

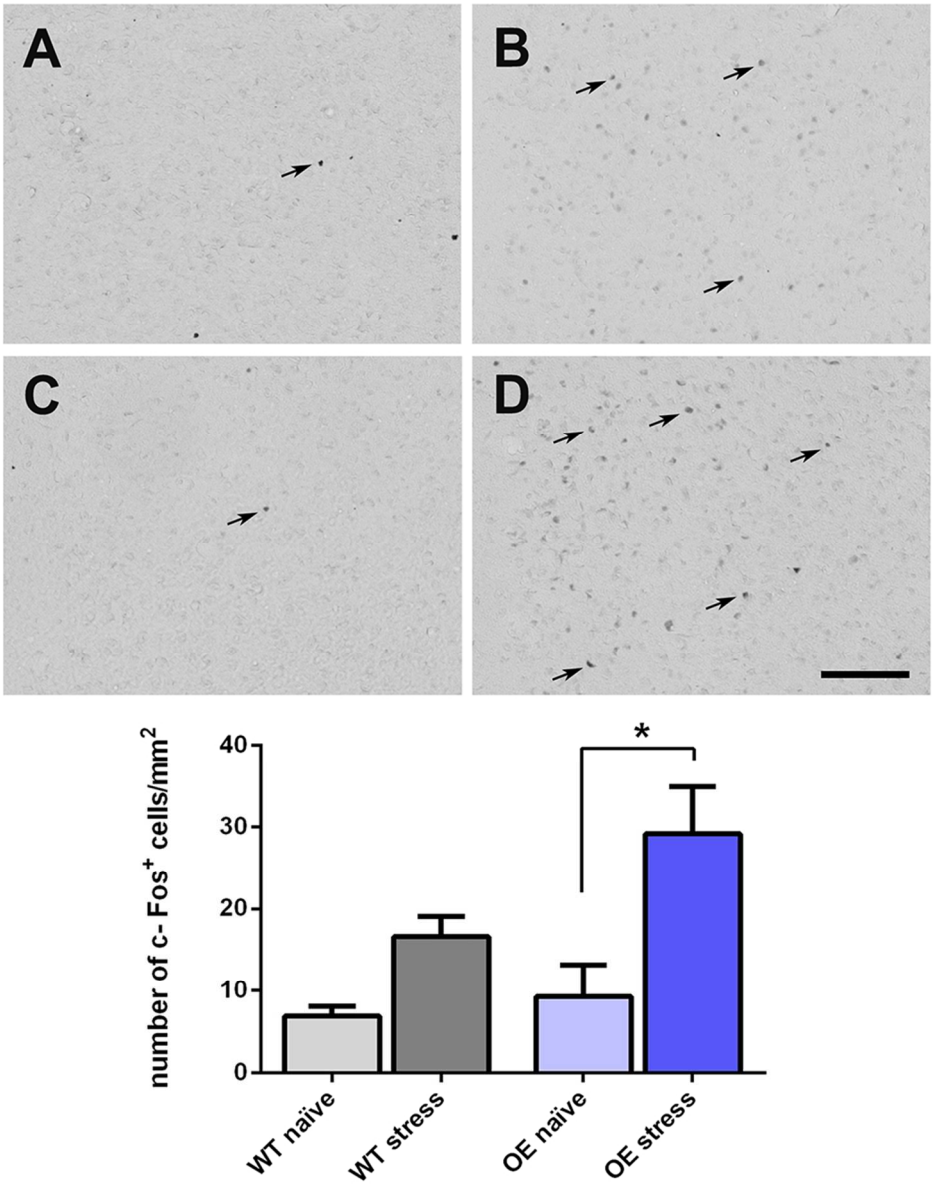

Figure 4

85x107mm (300 x 300 DPI)

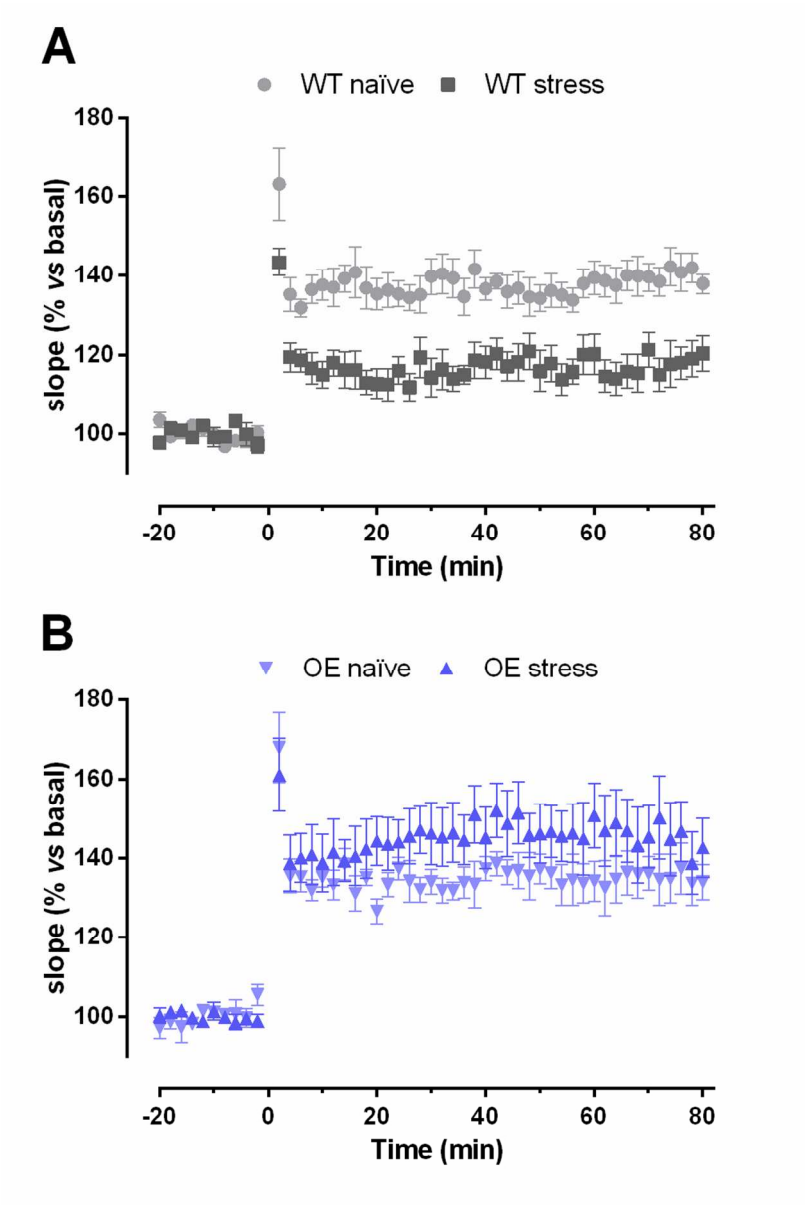

Figure 5

85x127mm (300 x 300 DPI)

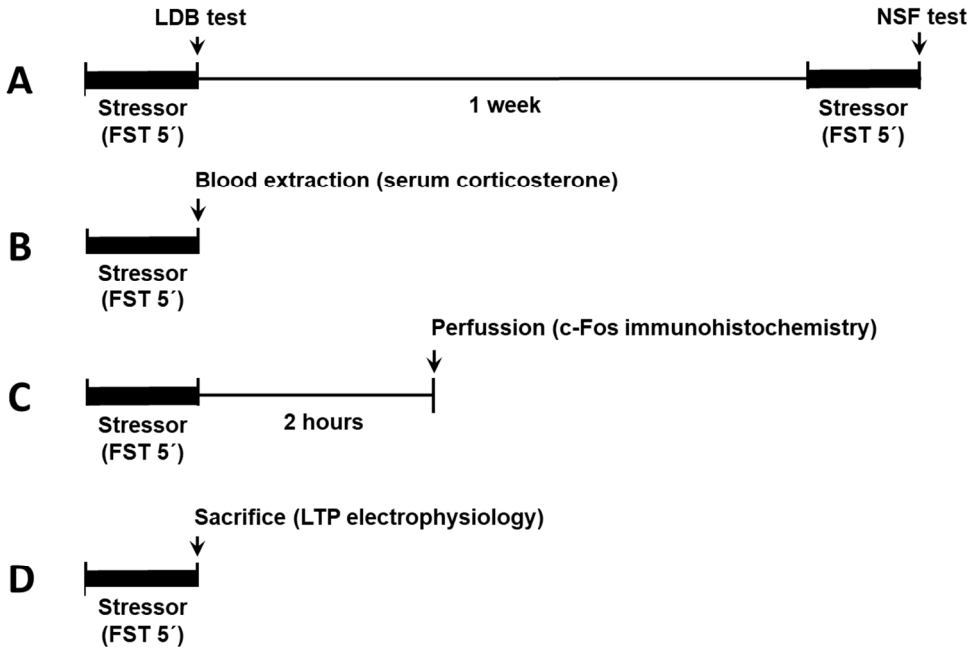

Figure 6

346x230mm (96 x 96 DPI)