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ENHANCED NORADRENERGIC ACTIVITY IN THE AMYGDALA CONTRIBUTES TO HYPERAROUSAL IN AN ANIMAL MODEL OF PTSD

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Summary

Increased activity of the noradrenergic system in the amygdala has been suggested to contribute to the hyperarousal symptoms associated with post-traumatic stress disorder (PTSD). However, only two studies have examined the content of noradrenaline or its metabolites in the amygdala of rats previously exposed to traumatic stress showing inconsistent results. The aim of this study was to investigate the effects of an inescapable foot shock (IFS) procedure 1) on reactivity to novelty in an open-field (as an index of hyperarousal), and 2) on noradrenaline release in the amygdala during an acute stress. To test the role of noradrenaline in amygdala, we also investigated the effects of microinjections of propranolol, a β-adrenoreceptor antagonist, and clenbuterol, a β-adrenoreceptor agonist, into the amygdala of IFS and control animals. Finally, we evaluated the expression of mRNA levels of β-adrenoreceptors (β1 and β2) in the amygdala, the hippocampus and the prefrontal cortex. Male Wistar rats (3 months) were stereotaxically implanted with bilateral guide cannulae. After recovering from surgery, animals were exposed to IFS (10 shocks, 0.86 mA, and 6 seconds per shock) and seven days later either microdialysis or microinjections were performed in amygdala. Animals exposed to IFS showed a reduced locomotion compared to non-shocked animals during the first 5 minutes in the open-field. In the amygdala, IFS animals showed an enhanced increase of noradrenaline induced by stress compared to control animals. Bilateral microinjections of propranolol (0.5 µg) into the amygdala one hour before testing in the open-field normalized the decreased locomotion observed in IFS animals. On the other hand, bilateral microinjections of clenbuterol (30 ng) into the amygdala of control animals did not change the exploratory activity induced by novelty in the open field. IFS modified the mRNA expression of β1 and β2 adrenoreceptors in the prefrontal cortex and the hippocampus. These results suggest that an increased noradrenergic activity in the amygdala contributes to the expression of hyperarousal in an animal model of PTSD.

Keywords: PTSD, noradrenaline, amygdala, propranolol, clenbuterol, hyperarousal

1. Introduction

Post-traumatic stress disorder (PTSD) is an anxiety disorder that results from experiencing an extremely traumatic event and is defined as a long term, maladaptive stress response (Pitman et al., 2012; Yehuda et al., 2015). PTSD is characterized in part by symptoms of hyperarousal resulting from a non-associative general sensitization process (Dunsmoor and Paz, 2015; Pitman et al., 2012; Stam, 2007). Also, heightened heart rate reactivity to startling stimuli (loud tones) and larger skin conductance response to novel stimuli have been observed in PTSD patients (Pitman et al., 2012). In animal models, exaggerated acoustic startle response and reduced locomotor activity in a novel environment have been used as measures of hyperarousal after exposure to traumatic stressors (Hendriksen et al., 2010; Kinn Rød et al., 2012; Stam, 2007; van Dijken et al., 1992; Wang et al., 2012).

Noradrenaline has a special role in mediating arousal and emotional memories, and is also involved in fear responses (Roozendaal and McGaugh, 2011; Sara, 2009). It has been proposed that an altered noradrenergic activity may contribute to the hyperarousal symptoms associated with PTSD (Krystal and Neumeister, 2009; O'Donnell et al., 2004; Southwick et al., 1999; Strawn and Geracioti, 2008). This proposal is based on studies showing that the reduction and increase of noradrenergic activity attenuated and precipitated, respectively, some of the symptoms in PTSD patients (Boehnlein and Kinzie, 2007; Bremner et al., 1997; Raskind et al., 2007; Southwick et al., 1993; Taylor et al., 2008). Additionally, the systemic treatment with pharmacological agents that reduce noradrenergic transmission normalized acoustic startle response in mice previously exposed to inescapable foot-shock (IFS) (Olson et al., 2011). Moreover, the evoked responses to a noxious event are higher in neurons of the locus coeruleus (primary source of noradrenaline in the forebrain) of rats previously exposed to single prolonged stress (George et al., 2013).

The amygdala is a key area involved in the recognition of dangerous stimuli and the coordination of fear response (LeDoux, 2007; Roozendaal and McGaugh, 2011). Indeed, it has been reported exaggerated amygdala activation in response to trauma-related stimuli as well as generic (neutral) stimuli in patients with PTSD compared to control subjects (Pitman et al., 2012; Rauch et al., 2006). Therefore, an increased stress-related noradrenergic activity in amygdala may contribute to the expression of hyperarousal symptoms in PTSD. Despite this, and to our knowledge, there are only two studies examining the content of noradrenaline or its metabolites in the amygdala of rats previously exposed to traumatic stress showing inconsistent results (Hendriksen et al., 2010; Tsuda et al., 1986). Moreover, no studies have monitored the *in vivo* release of noradrenaline in the amygdala or determined the causal relationship between these changes of noradrenaline outflow and the alterations of behavior in animals exposed to traumatic stress. Although the amygdala seems to be the main area upon which noradrenaline exerts its effects, other areas mediating fear processing such as prefrontal cortex and hippocampus may also be involved (Pitman et al., 2012).

To investigate whether an enhanced noradrenergic activity in the amygdala is involved in PTSD-like behavioral changes, we exposed male Wistar rats to an IFS procedure. This paradigm induces both long-lasting conditioned and non-conditioned anxiety (Chen et al., 2012; Daviu et al., 2010; Hendriksen et al., 2010; Hendriksen et al., 2012; Kinn Rød et al., 2012; van Dijken et al., 1992). In particular, IFS procedure produces a reduction in the activity in unknown environments 7 days after exposure to the shock (Daviu et al., 2010; Hendriksen et al., 2010; van Dijken et al., 1993; van Dijken et al., 1992). This effect of IFS has been considered an index of hypervigilance similar to the hyperarousal observed in PTSD patients (Pitman et al., 2012; Stam, 2007).

The specific aims of this study were to investigate: 1) the effect of IFS on the reactivity to novelty in an open-field (as an index of hyperarousal) and the noradrenaline release in the

amygdala during an acute restraint stress; 2) the effect of the amygdala injection of the β-adrenoceptor antagonist propranolol on the changes in the reactivity to novelty produced by IFS; 3) the effect of the amygdala injection of the β-adrenoceptor agonist clenbuterol in control rats; and 4) the expression of mRNA levels of β-adrenergic (β1 and β2) receptors in the amygdala, the hippocampus and the prefrontal cortex of control and IFS rats.

2. Methods and Materials

2.1 Animals

Young adult male Wistar rats (Harlan, The Netherlands) were 4 weeks old (125-150 g) upon arrival. Experimental procedures started 2 months later. Animals were housed (2 animals per cage) and provided with food and water *ad libitum* and maintained in a temperature-controlled room (22 ±2 ºC) under an inverted light/dark cycle (lights on at 20:00 – 8:00). The experiments were carried out during the dark phase of the cycle between 14:00 and 19:00. Three different sets of animals were used for a) evaluation of the behavioral effects of IFS procedure and mRNA quantification, b) microdialysis experiments, and c) microinjection experiments (Figure 1). All experiments carried out in our laboratory at the Universidad Complutense of Madrid followed the Spanish regulations for the protection of laboratory animals (RD53/2013).

2.2 Inescapable Foot-Shock (IFS) procedure

Rats were place in a shuttle-box divided into 2 compartments separated by a guillotine door. The starting compartment (light compartment, 50x50x20 cm) consisted on an open roof white plastic compartment illuminated by a 60 W bulb at 40 cm overhead. The shock compartment (dark compartment, 25x25x20 cm) was made of black plastic, a removable roof, no illumination, and an electrified grid floor. Rats were first place in the light compartment for 60 seconds and then the guillotine door was opened. Once the animal entered in the dark compartment the door was closed and 10 shocks (0.86 mA) of 6 seconds duration in 10 minutes were given. The conditions of the IFS procedure were based in previous studies (Hendriksen et al., 2010; Hendriksen et al., 2012; van Dijken et al., 1993; van Dijken et al., 1992). Control animals remained 10 minutes in the dark compartment without receiving shocks. Rats were then placed back in their home cage during 7 days before experiments started. All animals were handled three times for 1 min before the exposure to the shuttle-box/IFS procedure in order to habituate them to the experimenter.

2.3 Open field test

Behavioral responses towards a novel context not associated with the IFS procedure were evaluated in open field arenas (MED Associates INC., St. Albans, USA). The open field apparatus consisted of a Plexiglas box (80x80x30 cm) equipped with two horizontal rows of eight infrared light sensitive photocell beams located at 5 and 15 cm, respectively, from the basement, allowing the detection of horizontal and vertical (rearing) motor activity. Interruptions of the photocell beams were registered automatically by computer software connected to the open field apparatus (MED Associates Inc., St. Albans, USA). Open field arenas were wiped with 70% ethanol between rats. Animals were placed in the center of the arena and activity was recorded every 5 min for a total time of 60 min. IFS-exposed rats spent more time in the center because of the reduction in the activity induced by the shocks (data not shown).

2.4 Implantation of guide cannulae

The procedures for the microinjections and microdialysis experiments were adapted from previous studies of the laboratory (del Arco et al., 2015; Ronzoni et al., 2016). Animals were anesthetized with equithesin (2.5 mg/kg i.p.) and received a subcutaneous dose of the local anesthetic lidocaine in the incision area (20 mg/ml) and the non-steroidal analgesic carprofen (4 mg/kg, i.m.) before been positioned in the stereotaxic apparatus (Kopf Instruments). Bilateral guide cannulae were implanted to reach the amygdala with the following coordinates according to Paxinos and Watson, 1998: -3.1 mm caudal, ±5 mm medial and -5.5 or -6.5 mm (respectively for microdialysis and microinjection experiments) from the top of the skull, being the incisive bar set at -3.3 mm) (Paxinos and Watson, 1998). Stainless-steel guide cannulae [made in our own workshop], for microdialysis experiments (10 mm; 20-gauge) or for microinjections (15mm; 23-gauge) experiments were fixated to the skull with 2-3 anchoring screws (Angtho´s, Sthockholm, Sweden) and dental acrylic cement. Stainless-steel dummy cannulae (24-gauge) were inserted into the guide to keep it clean and prevent occlusion. After surgery, the rats received a subcutaneous injection of 3 ml of saline to

facilitate clearance of drugs and prevent dehydration. One day after surgery rats were again group-housed two per cage and allowed to recover for a minimum of 7 days before submission to the IFS procedure. Wound from surgery, weight and general welfare of animals were monitored.

2.5 Microdialysis experiments

Microdialysis probes, [constructed in our own workshop], were of concentric design with an active dialysis membrane (5000 Da, Hospal, Barcelona,Spain) of 2 mm in length. Thus, the whole amygdala was perfused during the experiments (figure 3B). One day before the experiments, all animals were habituated to the mycrodialysis cages. In the experimental day, probes were perfused with artificial cerebrospinal fluid (CSF) consisting of (in mM): NaCl 137; $CaCl_2$ 2.4; KCl 3; $MgSO_4$ 0.5; Na_2HPO_4 2; glucose 3; containing the inhibitor of noradrenaline transporter nomifensine (5 µM) at a flow rate of 2 µl/min. After basal concentrations of neurotransmitter were established (3 h perfusion period), 20 min samples were collected and immediately stored at -80º C until analyzed. The first three samples were used as a control (basal levels) and then it followed an acute stress period (40 min of restrain stress). Acute stress consisted of restraint by tightly wrapping the rat using a cloth tied with Velcro.

2.6 HPLC analysis of noradrenaline

Noradrenaline was analyzed by reverse-phase HPLC and electrochemical detection (HP1049A, Agilent, Palo Alto, USA). Samples were injected in a Rheodyne injector (20 µl loop) running in a C18 column of 4 µm particles, and 3.9x150 mm (Nova-pack, waters, Milford, MA). The mobile phase consisted of 0.1 M acetate-citrate buffer (pH = 3.2 adjusted with HCl and NaOH 1 N), 1 mM EDTA, 9 mM sodium octyl sulphonate, and 15% methanol. The mobile phase was re-circulated at a flow rate of 1 ml/min. These conditions allowed noradrenaline to be detected at 3.05 min. Noradrenaline was measured by a coulometric detector (Coulochem II model 5200, ESA). Conditioning cell (ESA 5011) was set at 0 mV and

analytical cells at +375 mV (cell 1) and -250 mV (cell2). Chromatograms were processed using the Millenium software (waters). The limit of detection for noradrenaline (20 µl samples) was 0.15 nM.

2.7 Microinjections into the amygdala and drugs

Bilateral microinjections into de amygdala were performed using 30-gauge stainless steel injection cannulae, protruding 2 mm below the tip of the guide and attached to a 10 µl Hamilton microsyringe. By means of a micropump (Hardvard Apparatus, Holliston, MA, USA) an injection volume of 0.5 µl/side at a rate flow of 0.5 µl/min was injected maintaining the injection cannulae in place for 60 seconds to allow the diffusion of the drug/vehicle. The injection conditions, in particular the injection volume, allows the drugs to diffuse into the basolateral amygdala and surrounding amygdalar nuclei. The nonspecific β-adrenoceptor antagonist propranolol (Sigma-Aldrich, Spain) (0.1 µg, 0.5 µg and 1.5 µg per side) and the β2-adrenoceptor agonist clenbuterol (Sigma-Aldrich, Spain) (30 ng per side) were freshly dissolved in CSF that was also used as vehicle treatment, and injected 1 hour before placing the animal in the open field arena. Drug doses were based on previous studies performing microinjections into the amygdala and were within the range of those used to modulate emotional memory (Introini-Collison et al., 1995; LaLumiere and McGaugh, 2005; Roozendaal et al., 2008).

2.8 Histology

All animals used for microdialysis and microinjection experiments were anesthetized with an overdose of sodium pentobarbital (100 mg/kg i.p.) and perfused intracardially with 0.9% saline followed by 4% formaldehyde. The brains were removed and immersed in fresh 4% formaldehyde and then submerged in a 25% sucrose (wt/vol) solution in water for cryoprotection. Coronal sections of 50 µm were cut in a cryostat (Leica CM1510S) mounted in gelatin –coated slides, and stained with cresyl violet. The sections were examined and the

placement of the microdialysis probes and the injections cannulae was verified under a light microscope (Axioskop, Zeiss, Alemania) (figure 3 A and C).

2.9 Real-time PCR

For mRNA determination in brain tissue, rats were killed 2-3 days after open field test by decapitation between 9:00 and 11:00 and brains were immediately frozen by isopentane and dry ice and stored at -80 ºC. Punches from ventral-medial prefrontal cortex (including prelimbic and infralimbic subregions), dorsal hippocampus and amygdala were collected and stored again at -80º C. Total RNAs were purified from tissue by the single step procedure of Chomczynski and Sacchi (1987) using Tri-Reagent (Sigma, Spain) (Chomczynski and Sacchi, 1987). The concentration and purity of RNA extracted was determined by an automated electrophoresis system (Experion, Bio-Rad, USA). One microgram of total RNA extracted from the PFC tissue was reversed transcribed into first strand complementary DNA using GoScript Reverse Transcription system (Promega Biotech Ibérica, Spain). Real-time PCR was performed in iQ5 equipment using the SsoFast EvaGreen Supermix (Bio-Rad, USA) and 500 nM concentrations of specific primers. The sequences of the oligonucleotide primers used to amplify β1- and β2-adrenoceptors were as follows: β1: 5´GCAGAACCAGTCTCACAGCTAA and 3´CGCCTTTCTACCTCTAGTGCAT; β2: 5´CTCCTTAACTGGTTGGGGCTATG and 3´TCCCATAGGTTTTCGAAGAAGA. The amount of targets, normalized to an endogenous reference (18S ribosomic mRNA) and relative to a calibrator, was defined by the threshold cycle (Ct) methods (Livak and Schmittgen, 2001). In all runs, melting curves were performed to make sure that only the corresponding DNA fragment was amplified.

2.10 Statistical analysis

To analyze motor activity and dialysate concentration of noradrenaline, a two-way analysis of variance (ANOVA) design, with repeated measures when appropriate, was used to perform planned comparisons (*a priori* analysis), considering group (control or IFS) and time or drug

(propranolol or clenbuterol) as within- and between-subject factors, respectively. For the analysis of noradrenaline dialysate concentrations, samples collected prior to acute stress were average to yield a standard baseline level of 100%. Stress effect in noradrenaline was expressed as the percent change from these values. Student´s *t* test for independent samples was performed to analyze mRNA receptor quantification and the effects of clenbuterol injections. Statistical analyses were performed with STATISTICA software. Statistical signification was considered in all cases $p < 0.05$.

3. Results

3.1 Effects of IFS in the exploratory activity in response to novelty.

Total (60 min) exploratory activity in response to novelty was not significantly different between IFS and control animals (locomotion: $F_{(1,17)}$= 4.44, p= 0.05; rearing: $F_{(1,17)}$= 0.28, p= 0.60) although there was a significant interaction between group and time factors (locomotion: $F_{(11,187)}$= 10.33, p< 0.001; rearing: $F_{(11,187)}$= 3.02, p= 0.001). IFS induced a lower exploratory activity in response to novelty during the first 5 minutes of the test compared to control animals (locomotion: $F_{(1,17)}$= 92.94, p <0.001; rearing: $F_{(1,17)}$= 20.76, p <0.001) (Figure 2).

3.2 Effect of IFS on the increase of extracellular concentration of noradrenaline in the amygdala induced by acute restraint stress.

Basal dialysate concentration of noradrenaline in the amygdala (see cannulae location in figure 3A and B) of IFS animals were significantly lower than in control animals (control= 0.83 ± 0.09 nM; IFS= 0.39 ± 0.07 nM, $F_{(1,19)}$= 14.96, p= 0.001).

The effect of stress on the dialysate concentration of noradrenaline was calculated as the average of 80-120 min time samples. As shown in figure 4, an acute restraint stress increased significantly the dialysate concentration of noradrenaline in the amygdala of IFS (212.84 ± 13.72 %, $F_{(1,19)}$= 27.48, p< 0.001) but not control (127.33 ± 2.67%, $F_{(1,19)}$= 1.21, p= 0.29) animals. There was a significant difference between IFS and control animals in the effect of stress on the dialysate concentration of noradrenaline in the amygdala ($F_{(1,19)}$= 6.76, p= 0.018).

3.3 Effects of propranolol and clenbuterol microinjections into the amygdala on the exploratory activity in response to novelty.

For the study of the effects of amygdala microinjections, exploratory activity during the first 5 minutes in the open field was analyzed. A two-way ANOVA showed a significant interaction between drug treatment (propranolol 0.5 µg *vs* vehicle) and IFS treatment (IFS *vs* control)

(locomotion: $F_{(1,29)}$= 30,55, p< 0.001; rearing: $F_{(1,28)}$= 5.46, p= 0.027). There was also a significant effect of the factor IFS treatment on novelty induced locomotion ($F_{(1,29)}$= 29.39, p< 0.001) and rearing ($F_{(1,28)}$= 26.75, p< 0.001). As shows in figure 5, propranolol 0.5 µg injected into the amygdala of control animals reduced locomotion ($F_{(1,29)}$= 11.78, p= 0.002) but not rearing ($F_{(1,28)}$= 0.15, p=0.706). Propranolol microinjections at a dose of 0.5 µg in IFS animals significantly reversed the reduction in locomotion ($F_{(1,29)}$= 19.36, p< 0.001) and rearing ($F_{(1,28)}$= 8.50, p= 0.007) observed in the IFS animals treated with vehicle. The effects of injections of propranolol 0.1 or 1.5 µg in the amygdala of IFS animals were not significantly different from injections of propranolol 0.5 µg (Table 1).

As shows in Table 1, clenbuterol (30 ng) injected into the amygdala of control animals did not change the exploratory activity (5 first minutes) induced by novelty in the open field (locomotion $t_{(13)}$= 0.77, p=0.453; rearing $t_{(13)}$= 1.11, p= 0.287).

3.4 Effect of IFS on mRNA levels of β-adrenoceptors (β_1 and β_2) in the amygdala, ventral-medial prefrontal cortex and dorsal hippocampus.

As shown in Table 2, IFS did not significantly modify the mRNA levels of β-adrenoceptors in the amygdala (β_1: $t_{(17)}$= 1.77,p= 0.095; β_2: $t_{(16)}$: 0.12, p= 0.908) nor in the medial prefrontal cortex (β_1: $t_{(15)}$= 0.91, p= 0.378; β_2: $t_{(14)}$: 2.03 , p=0.062) but enhanced the mRNA levels of β-adrenoceptors in the dorsal hippocampus (β_1: $t_{(13)}$= 2.52, p= 0.026; β_2: $t_{(13)}$= 2.25, p= 0.043).

4. Discussion

The aim of the present study was to investigate whether an enhanced noradrenergic activity in the amygdala is involved in the hyperarousal symptoms observed in PTSD. For that, we exposed male Wistar rats to IFS and a week later we measured reduced locomotion in a novel environment as an index of PTSD-like hyperarousal (Hendriksen et al., 2010; Hendriksen et al., 2012; van Dijken et al., 1992). This study shows for the first time that exposure to IFS enhances stress-induced noradrenaline extracellular concentration in the amygdala and that the blockade of β-adrenoceptors through local injections of propranolol counteracts the reduced exploratory activity produced by IFS. These findings suggest that IFS causes a sensitization of the noradrenergic projections to the amygdala that mediates, at least in part, the behavioral sequelae of IFS (hyperarousal).

Several findings support the hypothesis that an enhanced noradrenergic activity in the amygdala is involved in hyperarousal symptoms of PTSD patients (Krystal and Neumeister, 2009; O'Donnell et al., 2004; Southwick et al., 1999; Strawn and Geracioti, 2008). First, the amygdala is a key structure mediating emotional arousal and the effects of stress hormones on cognitive function (LeDoux, 2007; Roozendaal and McGaugh, 2011). As well, an exaggerated activation of the amygdala in response to trauma-related, and also neutral, stimuli has been reported in PTSD patients (Pitman et al., 2012; Rauch et al., 2006). Second, noradrenaline release in the amygdala seems to play a critical role in mediating the effects of emotional arousal on learning and memory (Roozendaal and McGaugh, 2011; Sara, 2009). Moreover, the reduction and increase of noradrenergic activity attenuated and precipitated, respectively, some of the symptoms in PTSD patients (Boehnlein and Kinzie, 2007; Bremner et al., 1997; Raskind et al., 2007; Southwick et al., 1993; Taylor et al., 2008). Evidence of noradrenaline dysregulation have been also shown in several studies measuring noradrenaline in the urinary system and in plasma of PTSD patients (reviewed in (Strawn and Geracioti, 2008). However, noradrenaline levels in the periphery do not necessarily

mirror those in the brain since they are derived from largely disparate sources. Interestingly, Geracioti et al. found a higher CSF noradrenaline concentrations in men with PTSD than in healthy men and also a positive correlation between CSF noradrenaline levels and the severity of PTSD symptoms (Geracioti et al., 2001).

In the present study we used the microdialysis technique to measure noradrenaline levels in the amygdala of IFS rats. As shown in the results section, basal dialysate concentrations of noradrenaline in the amygdala were reduced in animals exposed to IFS. Moreover, the percentage increase of dialysate noradrenaline evoked by acute restraint stress was enhanced in the amygdala of IFS rats. These results are in accord with a previous study in which exposure of rats to a single prolonged stress lowered spontaneous activity but increased the evoked response of neurons in the locus coeruleus, the main source of noradrenaline in the forebrain (George et al., 2013). Moreover, the sensitization of the noradrenergic system has been also observed after chronic stress exposure (Adell et al., 1988; Jedema and Grace, 2002; Nisenbaum et al., 1991). The reduced basal levels of noradrenaline found in the present study may be the consequence of an enhanced sensitivity of α2-adrenergic inhibitory autoreceptors in noradrenaline neurons (Aghajanian and VanderMaelen, 1982; Strawn and Geracioti, 2008). However, this change in α2-adrenoreceptors cannot account for the enhanced release of noradrenaline after stress. Alternatively, the lower basal release of noradrenaline could be the result of an increased expression of the noradrenaline transporter in noradrenaline terminals (Miner et al., 2006). In turn, the basal low levels of noradrenaline could potentiate the stress induced release due to a lower inhibitory tone through autoreceptors. Added to this, it has been reported a reduced availability of noradrenaline transporter in the locus coerulerus of PTSD patients, which may result in exaggerated synaptic availability of noradrenaline in projection areas (Pietrzak et al., 2013). To our knowledge, there are only two previous studies examining the content of noradrenaline or its metabolites in the amygdala of rats previously exposed to traumatic stress showing inconsistent results (Hendriksen et al., 2010; Tsuda et al., 1986). Tsuda et al.

reported that noradrenaline turnover (evaluated by measuring the levels of the noradrenaline metabolite MHPG-SO4) induced by psychological stress is enhanced in the amygdala of rats previously exposed to foot-shock (Tsuda et al., 1986). Hendriksen et al have shown no changes of noradrenaline levels in the amygdala, prefrontal cortex and hippocampus following IFS (Hendriksen et al., 2010). In contrast, in the present study we describe the differential dynamics of basal and stimulated noradrenaline outflow in the amygdala of IFS and control animals. These changes of noradrenaline release in the amygdala may mediate the behavioral effects of the exposure to IFS.

The hypothesis of an exacerbated noradrenergic activity in the amygdala being responsible of hyperarousal symptoms of PTSD is further supported by the finding that microinjections of the β-adrenoreceptor antagonist propranolol in the amygdala counteract the reduced exploratory activity produced by IFS. Interestingly, since propranolol significantly reduced locomotion in control animals, the increase in activity observed in IFS rats seems to be a specific action of propranolol blocking the effects of an enhanced noradrenergic transmission in the amygdala. This result is in line with a previous study showing that systemic treatment with pharmacological agents that reduce noradrenergic transmission normalized startle response in mice exposed to IFS (Olson et al., 2011). Interestingly, there are a number of case reports suggesting that propranolol may ameliorate hyperarousal in PTSD patients who have had only partial response to other therapies (Strawn and Geracioti, 2008). In order to know whether an increase in noradrenergic transmission in the amygdala is enough to induce hyperarousal, we locally injected the specific β2-adrenoreceptor agonist clenbuterol before exposing control animals to an unknown environment (open field arena). Microinjections of clenbuterol in the amygdala of control animals did not change their exploratory activity. The dose of clenbuterol used here is in the range of those used to modulate memory when injected in the amygdala (Introini-Collison et al., 1995; LaLumiere and McGaugh, 2005). The results of clenbuterol injections suggest that the increased noradrenergic activity in the amygdala is not sufficient to induce hyperarousal. Alternatively,

the effects of a hypernoradrenergic state in the amygdala could be mediated by β1-adrenoreceptors since propranolol is a nonselective beta blocker. Also, an increased activity of other neurotransmitter systems in the amygdala such as the serotoninergic projections from the raphe (Bailey et al., 2013; Hendriksen et al., 2014; Pitman et al., 2012) might be necessary for the expression of hyperarousal symptoms.

Added to the increase in the release of noradrenaline in the amygdala, a change in the expression of adrenoreceptors could be involved in the effects of IFS on the activity in a novel environment. This would be in agreement with clinical studies demonstrating that PTSD patients show increased noradrenergic responsiveness that may contribute to symptomatology (Boehnlein and Kinzie, 2007; Southwick et al., 1993). Therefore, we evaluated the mRNA levels of β1- and β2-adrenergic receptors in the amygdala. Previous studies have shown that the alteration in the activity of the circuit prefrontal cortex-hippocampus-amygdala is involved in the pathophysiology of PTSD (Bailey et al., 2013; Hendriksen et al., 2014; Pitman et al., 2012). Thus, we evaluated the expression of the β-adrenergic receptors in these three areas of the brain (see table 2). IFS did not significantly modify mRNA levels of β-adrenergic receptors in the amygdala or the prefrontal cortex. However, β-adrenergic receptors were overexpressed in the hippocampus of IFS animals. First, these results suggest that the effects of noradrenaline in the amygdala inducing hyperarousal in IFS animals seem not to be mediated by an increase in the expression of β-adrenergic receptors. Interestingly, the enhanced expression of these receptors in the hippocampus suggests that increased noradrenaline transmission in this area of the brain could be involved in the pathophysiology of PTSD (Acheson et al., 2012; O'Donnell et al., 2004). As mentioned before, the microinjections of clenbuterol in the amygdala did not have any effect on the activity of control animals. Therefore, the above mentioned hypothesis would be in agreement with our results with clenbuterol.

Finally, the reduced locomotion and rearing activity in a novel environment (open-field arena) after exposure to IFS has been considered as an anxiogenic effect comparable to the hyperarousal observed in PTSD patients (Hendriksen et al., 2010; Hendriksen et al., 2012; van Dijken et al., 1992). However, it has been reported that ACTH levels are not changed after exposure to a novel environment in rats previously exposed to a session of three shocks, suggesting that the reduced locomotion in unknown environments is not linked to a marked increase in fear (Daviu et al., 2010). It is of interest the fact that increases of ACTH are produced when the rats are exposed to novel stressors when longer-lasting sessions of shocks were used (van Dijken et al., 1993). Daviu et al also showed that the reduced activity to novel environments is not observed when fear to the original shock context was prevented by shocking the animals immediately after exposure to the IFS-apparatus (Daviu et al., 2010). Interestingly, Siegmund and Wotjak reported that the pharmacological reduction of the conditioned-fear response to the shock context did not modify the fear response to a neutral tone in a new context, suggesting that hyperarousal depends on sensitization induced by IFS (Siegmund and Wotjak, 2007). These findings suggest that the reduced activity induced by IFS seems to be a conditioned behavior that can be confounded with hyperarousal. As we report here, IFS does not produce a general hypoactivity since the reduction in locomotion and rearing is only observed during the first five minutes of the test. This latter finding highlights the strong translational value of the test in regard to the stress-sensitization as one of the core symptoms of PTSD, along with the exaggerated response to trauma cues (Chen et al., 2012; Hendriksen et al., 2012; Korem and Akirav, 2014; Siegmund and Wotjak, 2007).

In conclusion, IFS exposure in rats enhanced stress-induced noradrenaline extracellular concentration in the amygdala and reduced locomotion in a novel environment as an index of PTSD-like hyperarousal. Moreover, the blockade of β-adrenergic receptors through local injections of propranolol in the amygdala counteracted the reduced exploratory activity produced by IFS. To our knowledge, these results show for the first time that an increased

noradrenergic activity in the amygdala contributes to the expression of hyperarousal behavior in an animal model of PTSD.

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References

Acheson, D.T., Gresack, J.E., Risbrough, V.B., 2012. Hippocampal dysfunction effects on context memory: Possible etiology for posttraumatic stress disorder. Neuropharmacology 62, 674-685.

Adell, A., Garcia-Marquez, C., Armario, A., Gelpi, E., 1988. Chronic Stress Increases Serotonin and Noradrenaline in Rat Brain and Sensitizes Their Responses to a Further Acute Stress. J.Neurochem. 50, 1678-1681.

Aghajanian, G.K., VanderMaelen, C.P., 1982. alpha 2-adrenoceptor-mediated hyperpolarization of locus coeruleus neurons: intracellular studies in vivo. Science 215, 1394-1396.

Bailey, C., Cordell, E., Sobin, S., Neumeister, A., 2013. Recent Progress in Understanding the Pathophysiology of Post-Traumatic Stress Disorder. CNS Drugs 27, 221-232.

Boehnlein, J.K., Kinzie, J.D., 2007. Pharmacologic Reduction of CNS Noradrenergic Activity in PTSD: The Case for Clonidine and Prazosin. J.Psychiatr.Pract. 13, 72-78.

Bremner, J., Innis, R.B., Ng, C.K., 1997. POsitron emission tomography measurement of cerebral metabolic correlates of yohimbine administration in combat-related posttraumatic stress disorder. Arch.Gen.Psychiatry 54, 246-254.

Chen, X., Li, Y., Li, S., Kirouac, G.J., 2012. Early fear as a predictor of avoidance in a rat model of post-traumatic stress disorder. Behav.Brain Res. 226, 112-117.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal.Biochem. 162, 156-159.

Daviu, N., Fuentes, S., Nadal, R., Armario, A., 2010. A single footshock causes long-lasting hypoactivity in unknown environments that is dependent on the development of contextual fear conditioning. Neurobiol.Learn.Mem. 94, 183-190.

del Arco, A., Ronzoni, G., Mora, F., 2015. Hypofunction of prefrontal cortex NMDA receptors does not change stress-induced release of dopamine and noradrenaline in amygdala but disrupts aversive memory. Psychopharmacology 232, 2577-2586.

Dunsmoor, J.E., Paz, R., 2015. Fear Generalization and Anxiety: Behavioral and Neural Mechanisms. Biol.Psychiatry 78, 336-343.

George, S.A., Knox, D., Curtis, A.L., Aldridge, J.W., Valentino, R.J., Liberzon, I., 2013. Altered locus coeruleus–norepinephrine function following single prolonged stress. Eur.J.Neurosci. 37, 901-909.

Geracioti, T.D., Baker, D.G., Ekhator, N.N., West, S.A., Hill, K.K., Bruce, A.B., Schmidt, D., Rounds-Kugler, B., Yehuda, R., Keck, P.E., Kasckow, J.W., 2001. CSF Norepinephrine Concentrations in Posttraumatic Stress Disorder. Am.J.Psychiatry 158, 1227-1230.

Hendriksen, H., Bink, D.I., Daniels, E.G., Pandit, R., Piriou, C., Slieker, R., Westphal, K.G.C., Olivier, B., Oosting, R.S., 2012. Re-exposure and environmental enrichment reveal NPY-Y1 as a possible target for post-traumatic stress disorder. Neuropharmacology 63, 733-742.

Hendriksen, H., Olivier, B., Oosting, R.S., 2014. From non-pharmacological treatments for post-traumatic stress disorder to novel therapeutic targets. Eur.J.Pharmacol. 732, 139-158.

Hendriksen, H., Prins, J., Olivier, B., Oosting, R.S., 2010. Environmental Enrichment Induces Behavioral Recovery and Enhanced Hippocampal Cell Proliferation in an Antidepressant-Resistant Animal Model for PTSD. PLoS ONE 5, e11943.

Introini-Collison, I.B., Ford, L., McGaugh, J.L., 1995. Memory Impairment Induced by Intraamygdala β-Endorphin Is Mediated by Noradrenergic Influences. Neurobiol.Learn.Mem. 63, 200-205.

Jedema, H.P., Grace, A.A., 2002. Chronic Exposure to Cold Stress Alters Electrophysiological Properties of Locus Coeruleus Neurons Recorded In Vitro. Neuropsychopharmacology 28, 63-72.

Kinn Rød, A.M., Milde, A.M., Grønli, J., Jellestad, F.K., Sundberg, H., Murison, R., 2012. Long-term effects of footshock and social defeat on anxiety-like behaviours in rats: Relatinoships to pre-stressor plasma corticosterone concentration. Stress 15, 658-670.

Korem, N., Akirav, I., 2014. Cannabinoids Prevent the Effects of a Footshock Followed by Situational Reminders on Emotional Processing. Neuropsychopharmacology 39, 2709-2722.

Krystal, J.H., Neumeister, A., 2009. Noradrenergic and serotonergic mechanisms in the neurobiology of posttraumatic stress disorder and resilience. Brain Res. 1293, 13-23.

LaLumiere, R.T., McGaugh, J.L., 2005. Memory enhancement induced by post-training intrabasolateral amygdala infusions of β-adrenergic or muscarinic agonists requires activation of dopamine receptors: Involvement of right, but not left, basolateral amygdala. Learn.Mem. 12, 527-532.

LeDoux, J., 2007. The amygdala. Curr.Biol. 17, R868-R874.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. Methods 25, 402-408.

Miner, L.H., Jedema, H.P., Moore, F.W., Blakely, R.D., Grace, A.A., Sesack, S.R., 2006. Chronic Stress Increases the Plasmalemmal Distribution of the Norepinephrine Transporter and the Coexpression of Tyrosine Hydroxylase in Norepinephrine Axons in the Prefrontal Cortex. J.Neurosci. 26, 1571-1578.

Nisenbaum, L.K., Zigmond, M.J., Sved, A.F., Abercrombie, E.D., 1991. Prior exposure to chronic stress results in enhanced synthesis and release of hippocampal norepinephrine in response to a novel stressor. J.Neurosci. 11, 1478-1484.

O'Donnell, T., Hegadoren, K.M., Coupland, N.C., 2004. Noradrenergic mechanisms in the pathophysiology of post-traumatic stress disorder. Neuropsychobiology 50, 273-283.

Olson, V.G., Rockett, H.R., Reh, R.K., Redila, V.A., Tran, P.M., Venkov, H.A., DeFino, M.C., Hague, C., Peskind, E.R., Szot, P., Raskind, M.A., 2011. The Role of Norepinephrine in Differential Response to Stress in an Animal Model of Posttraumatic Stress Disorder. Biol.Psychiatry 70, 441-448.

Paxinos, G., Watson, C., 1998. The rat brain in stereotaxic coordinates. Academic Press, New York.

Pietrzak, R.H., Gallezot, J., Ding, Y., 2013. Association of posttraumatic stress disorder with reduced in vivo norepinephrine transporter availability in the locus coeruleus. JAMA Psychiatry 70, 1199-1205.

Pitman, R.K., Rasmusson, A.M., Koenen, K.C., Shin, L.M., Orr, S.P., Gilbertson, M.W., Milad, M.R., Liberzon, I., 2012. Biological studies of post-traumatic stress disorder. Nat.Rev.Neurosci. 13, 769-787.

Raskind, M.A., Peskind, E.R., Hoff, D.J., Hart, K.L., Holmes, H.A., Warren, D., Shofer, J., O'Connell, J., Taylor, F., Gross, C., Rohde, K., McFall, M.E., 2007. A Parallel Group Placebo Controlled Study of Prazosin for Trauma Nightmares and Sleep Disturbance in Combat Veterans with Post-Traumatic Stress Disorder. Biol.Psychiatry 61, 928-934.

Rauch, S.L., Shin, L.M., Phelps, E.A., 2006. Neurocircuitry Models of Posttraumatic Stress Disorder and Extinction: Human Neuroimaging Research–Past, Present, and Future. Biol.Psychiatry 60, 376-382.

Ronzoni, G., Antón, M., Mora, F., Segovia, G., del Arco, A., 2016. Infralimbic cortex controls the activity of the hypothalamus-pituitary-adrenal axis and the formation of aversive memory: Effects of environmental enrichment. Behav.Brain Res. 297, 338-344.

Roozendaal, B., McGaugh, J.L., 2011. Memory modulation. Behav.Neurosci. 125, 797-824.

Roozendaal, B., Castello, N.A., Vedana, G., Barsegyan, A., McGaugh, J.L., 2008. Noradrenergic activation of the basolateral amygdala modulates consolidation of object recognition memory. Neurobiol.Learn.Mem. 90, 576-579.

Sara, S.J., 2009. The locus coeruleus and noradrenergic modulation of cognition. Nat Rev Neurosci 10, 211-223.

Siegmund, A., Wotjak, C.T., 2007. Hyperarousal does not depend on trauma-related contextual memory in an animal model of Posttraumatic Stress Disorder. Physiol.Behav. 90, 103-107.

Southwick, S.M., Krystal, J.H., Morgan, C., 1993. ABnormal noradrenergic function in posttraumatic stress disorder. Arch.Gen.Psychiatry 50, 266-274.

Southwick, S.M., Bremner, J.D., Rasmusson, A., Morgan III, C.A., Arnsten, A., Charney, D.S., 1999. Role of norepinephrine in the pathophysiology and treatment of posttraumatic stress disorder. Biol.Psychiatry 46, 1192-1204.

Stam, R., 2007. PTSD and stress sensitization: A tale of brain and body. Part 2: Animal models. Neurosci.Biobehav.Rev. 31, 558-584.

Strawn, J.R., Geracioti, T.D., 2008. Noradrenergic dysfunction and the psychopharmacology of posttraumatic stress disorder. Depress.Anxiety 25, 260-271.

Taylor, F.B., Martin, P., Thompson, C., Williams, J., Mellman, T.A., Gross, C., Peskind, E.R., Raskind, M.A., 2008. Prazosin Effects on Objective Sleep Measures and Clinical Symptoms in Civilian Trauma Posttraumatic Stress Disorder: A Placebo-Controlled Study. Biol.Psychiatry 63, 629-632.

Tsuda, A., Tanaka, M., Ida, Y., Tsujimaru, S., Ushijima, I., Nagasaki, N., 1986. Effects of preshock experience on enhancement of rat brain noradrenaline turnover induced by psychological stress. Pharmacol.Biochem.Behav. 24, 115-119.

van Dijken, H.H., de Goeij, D.C., Sutanto, W., Mos, J., de Kloet, E.R., Tilders, F.J.H., 1993. Short Inescapable Stress Produces Long-Lasting Changes in the Brain-Pituitary-Adrenal Axis of Adult Male Rats. Neuroendocrinology 58, 57-64.

van Dijken, H.H., Mos, J., van der Heyden, J.A.M., Tilders, F.J.H., 1992. Characterization of stress-induced long-term behavioural changes in rats: Evidence in favor of anxiety. Physiol.Behav. 52, 945-951.

Wang, Q., Yu, K., Wang, J., Lin, H., Wu, Y., Wang, W., 2012. Predator stress-induced persistent emotional arousal is associated with alterations of plasma corticosterone and hippocampal steroid receptors in rat. Behav.Brain Res. 230, 167-174.

Yehuda, R., Hoge, C.W., McFarlane, A.C., Vermetten, E., Lanius, R.A., Nievergelt, C.M., Hobfoll, S.E., Koenen, K.C., Neylan, T.C., Hyman, S.E., 2015. Post-traumatic stress disorder. Nat.Rev.Dis.Primers 15057.

Figure Legends

Figure 1. Diagram showing the design of the 3 set of experiments performed: a) Evaluation of behavioral effects of IFS and mRNA quantification; b) Microdialysis experiments; and c) Microinjection experiments.

Figure 2. Effect of IFS on novelty-induced exploratory activity in an open field apparatus. The temporal profiles represent A) locomotion in cm and B) rearing in counts in IFS (n=9) and control (n=10) rats. Insets: Activity during the first 5 minutes. Data are presented as mean ± SEM. ***p<0.001 compared to control, according to planned comparisons analysis in a 2-way (group x time) ANOVA with repeated measures.

Figure 3. Hystochemical analysis. A) Representative photomicrograph illustrating the placement of the membrane probe in the BLA for microdialysis. B) Approximate location of the microdialysis membrane in the amygdala of all animals included in the analysis. C) Representative micrograph illustrating the placement of the cannula in the BLA for microinjections experiments. D) Approximate location of the injection cannula in the amygdala of all animals included in the analysis. Abbreviations of amygdala areas: BA - basal nucleus; BLA - basolateral region; LA - lateral nucleus; M - medial nucleus.

Figure 4. Effect of restraint stress (40 minutes) in IFS and control animals on extracellular concentration of noradrenaline in the amygdala of the rat. Data (mean ± SEM) represent the temporal profile as percentage of the baseline dialysate concentration of neurotransmitter. The number of animals is shown in parenthesis. *p< 0.05 compared to control animals, according to planned comparisons in a 2-way (group x time) ANOVA with repeated measures.

Figure 5. Effects of bilateral microinjections of the β-adrenergic receptors antagonist propranolol (0.5 µg) into the amygdala on the exploratory activity in response to novelty in

IFS and control animals. Upper graph shows the effects of vehicle and propranolol (Prop) microinjections in control (n=8) and IFS (n=8-9) animals on locomotion. Bottom graph shows the effects of vehicle and propranolol (Prop) microinjections in control (n=8) and IFS (n=8-9) animals on rearing activity. Data (mean ± SEM) are expressed in centimeters for locomotion and counts for rearing. $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ compared to vehicle; ***p< 0.01 compared to control, according to planned comparisons analysis in a 2-way ANOVA.

Tables

Table 1
Effects of microinjections of propranolol and clenbuterol in the amygdala on the exploratory activity induced by novelty in an open field.

IFS animals		**LOCOMOTION**	**REARING**
Vehicle (n=8)		363.23 ± 62.04	13.71 ± 2.68
Propranolol	0.1 µg (n=7)	635.91 ± 153.87	46.00 ± 7.09**
	0.5µg (n=9)	755.64 ± 84.66**	39.78 ± 6.13**
	1.5µg (n=6)	743.89 ± 77.18*	50.67 ± 9.21 ***
Control animals		**LOCOMOTION**	**REARING**
Vehicle (n=8)		1067.67± 35.66	60.94 ± 8.29
Clenbuterol 30 ng (n=7)		989.52 ± 94.72	72.43 ± 5.69

Data (mean ± SEM) are show in cm for locomotion and counts for rearing (vertical activity). *** p< 0.001, ** p< 0.01, *p< 0.05 compared to vehicle according to planned comparisons in a 1-way ANOVA.

Table 2
mRNA content of β1 and β2 adrenoreceptors in the amygdala, medial prefrontal cortex (mPFC) and dorsal hippocampus (dHC).

	AMYGDALA		**mPFC**		**dHC**	
	CONTROL (n= 8-10)	**IFS** (n= 8-9)	**CONTROL** (n= 9-10)	**IFS** (n= 8-9)	**CONTROL** (n= 7-8)	**IFS** (n= 7-8)
ADRβ1	1.00 ± 0.08	0.82 ± 0.06	1.00 ± 0.14	0.85 ± 0.05	1.00 ± 0.14	1.53 ± 0.15*
ADRβ2	1.00 ± 0.07	0.99 ± 0.07	1.00 ± 0.14	0.68 ± 0.07	1.00 ± 0.08	1.40 ± 0.15*

Data (mean ± SEM) are show in absolute values (arbitrary units). *p<0.05 compared to control. Student *t*-test.

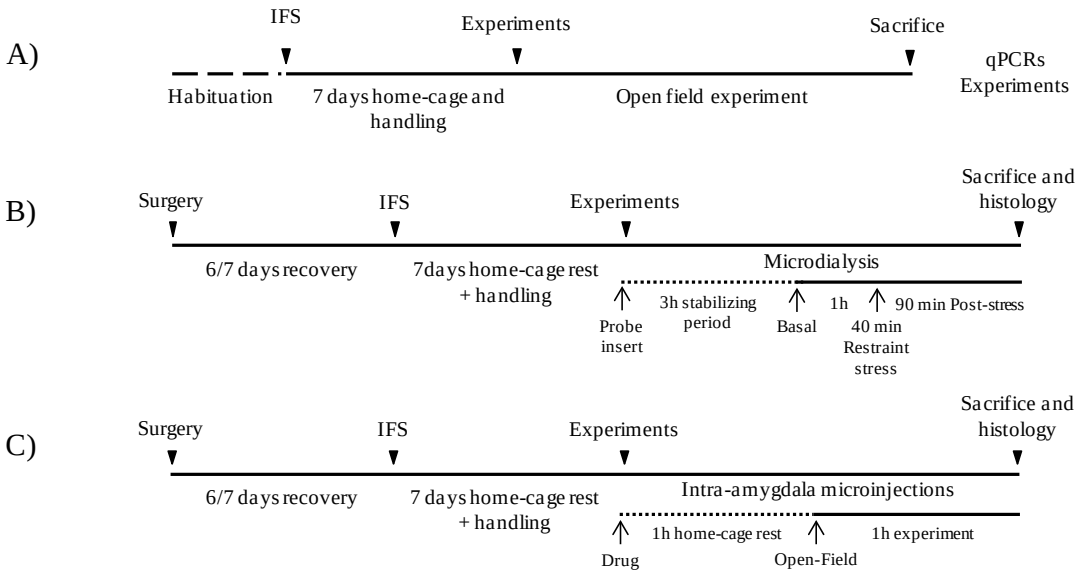
A)
IFS
Experiments
Sacrifice
qPCRs
Experiments
Habituation
7 days home-cage and handling
Open field experiment
B)
Surgery
IFS
Experiments
Sacrifice and histology
6/7 days recovery
7days home-cage rest + handling
Microdialysis
Probe insert
3h stabilizing period
Basal
1h
40 min Restraint stress
90 min Post-stress
C)
Surgery
IFS
Experiments
Sacrifice and histology
6/7 days recovery
7 days home-cage rest + handling
Intra-amygdala microinjections
Drug
1h home-cage rest
Open-Field
1h experiment

Figure 1

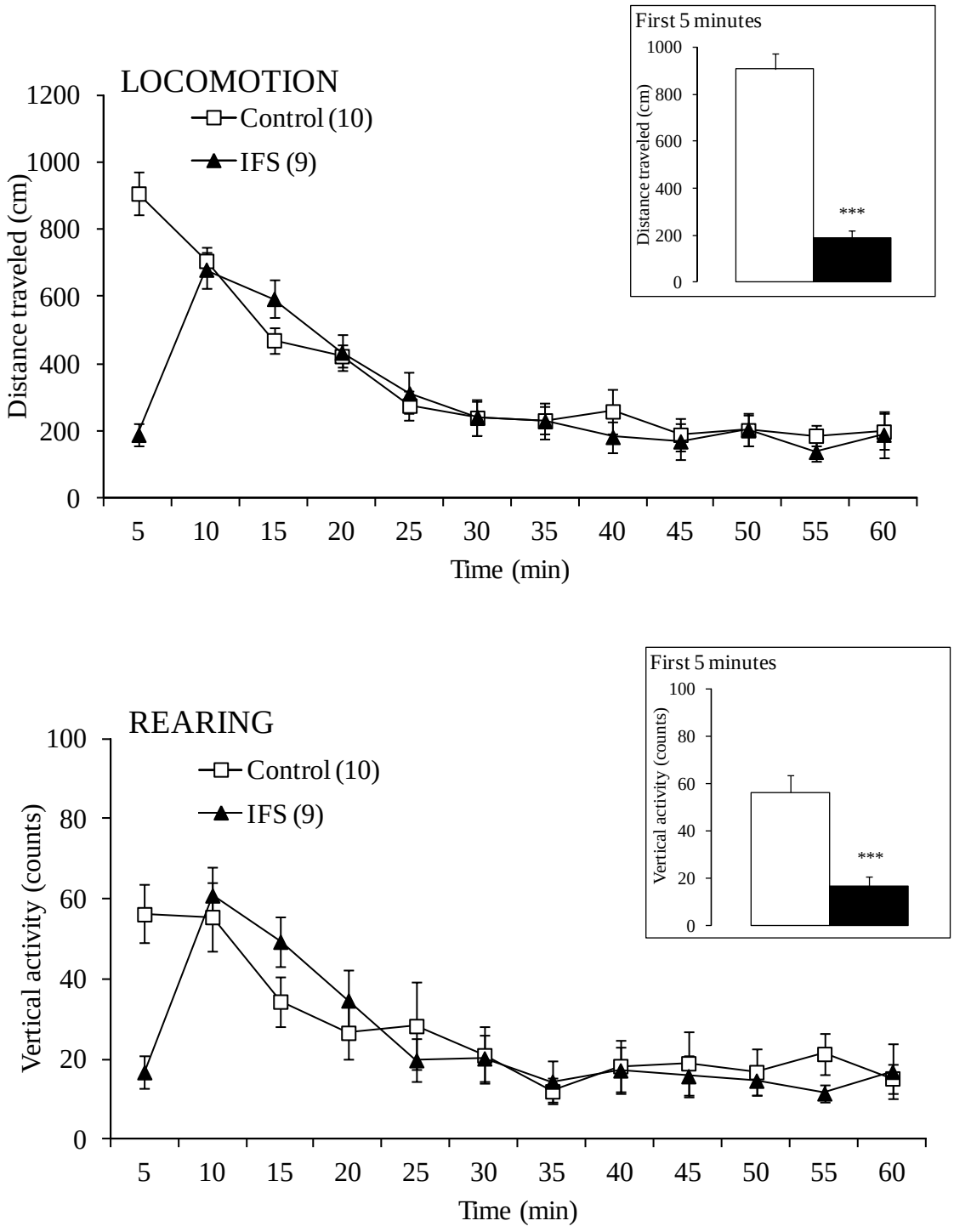
LOCOMOTION
Control (10)
IFS (9)
Distance traveled (cm)
Time (min)
First 5 minutes
Distance traveled (cm)
***
REARING
Control (10)
IFS (9)
Vertical activity (counts)
Time (min)
First 5 minutes
Vertical activity (counts)
***

Figure 2

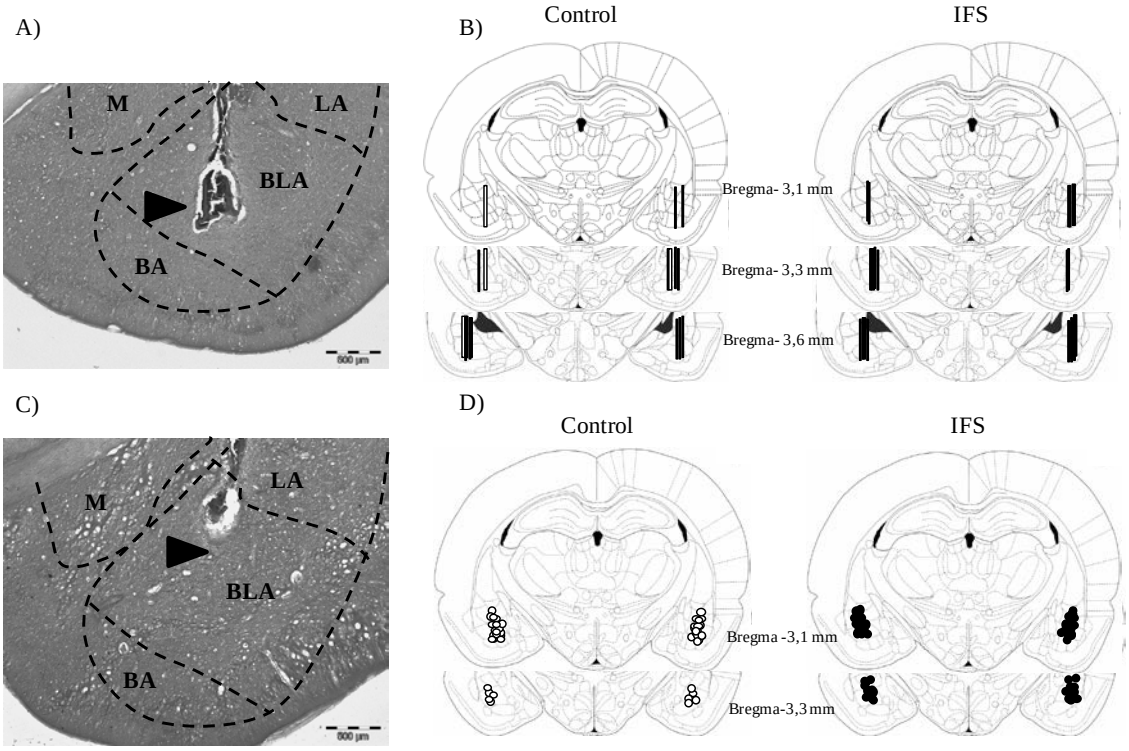
A)
M
LA
BLA
BA
B)
Control
IFS
Bregma- 3,1 mm
Bregma- 3,3 mm
Bregma- 3,6 mm
C)
M
LA
BLA
BA
D)
Control
IFS
Bregma -3,1 mm
Bregma-3,3 mm

Figure 3

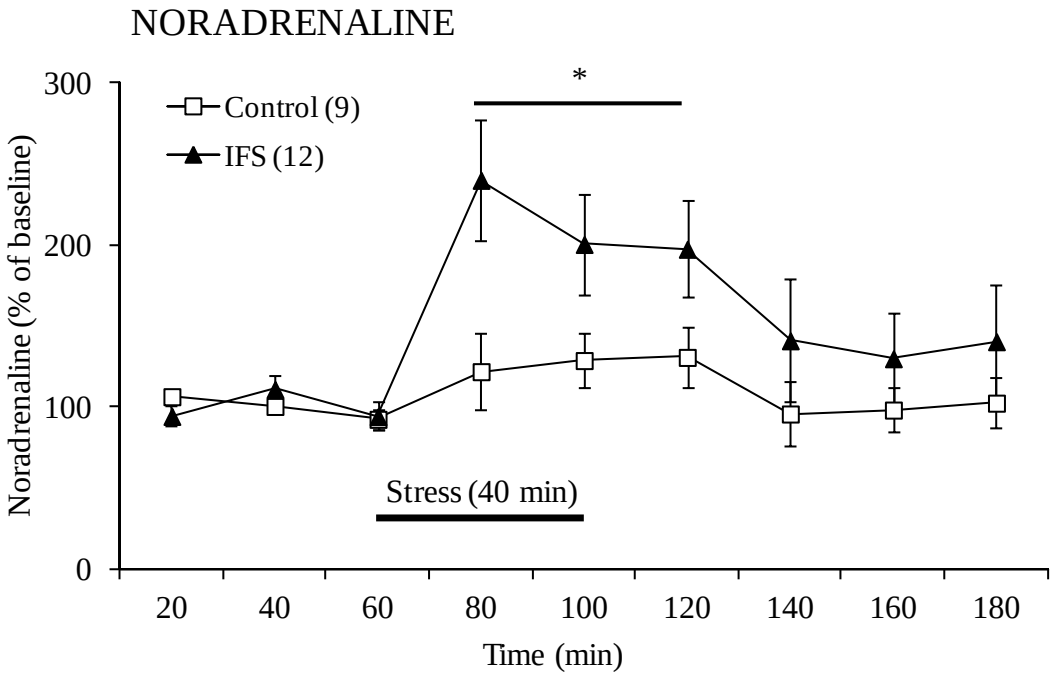
NORADRENALINE
Control (9)
IFS (12)
*
Noradrenaline (% of baseline)
300
200
100
0
Stress (40 min)
20
40
60
80
100
120
140
160
180
Time (min)

Figure 4

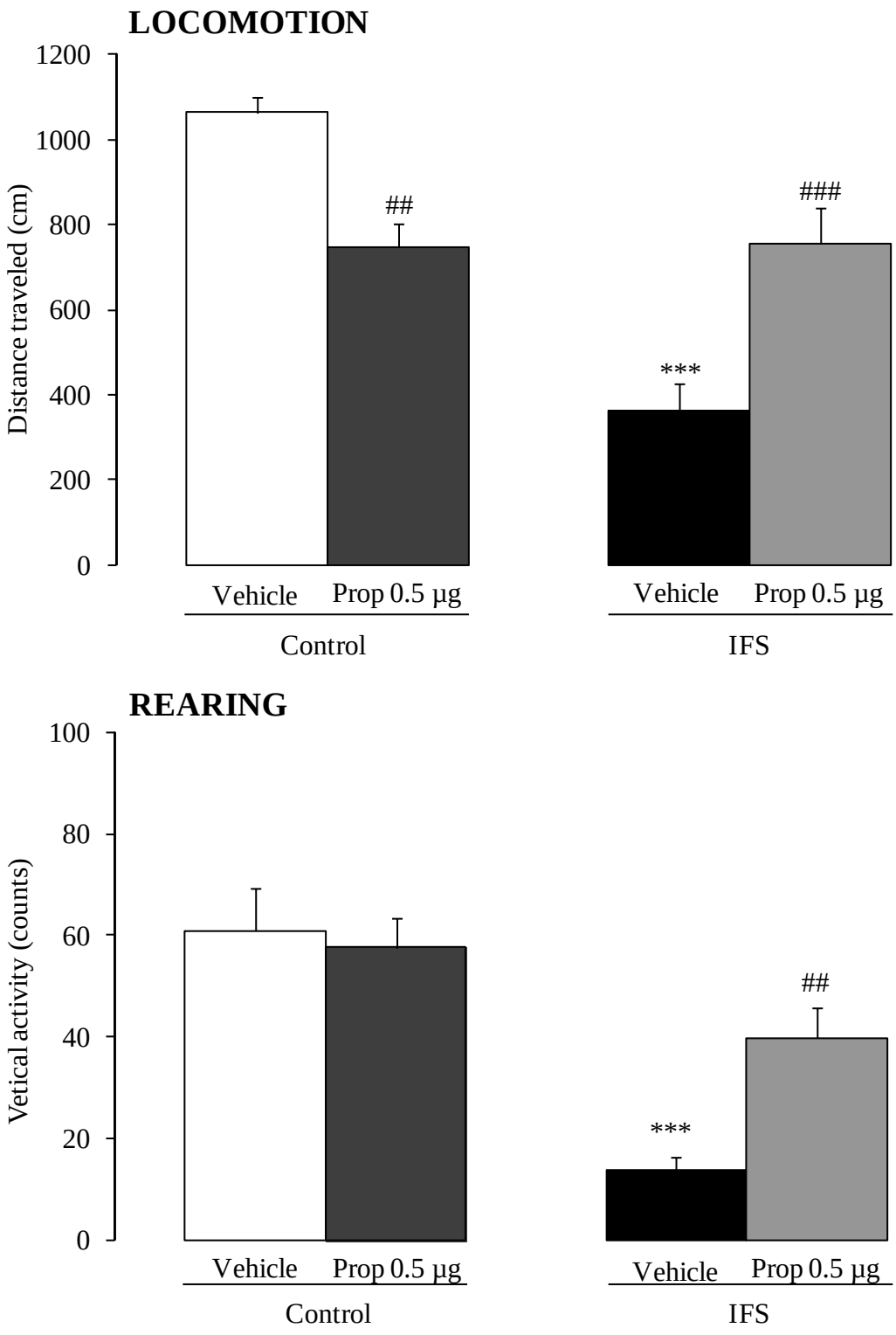
LOCOMOTION
Distance traveled (cm)
1200
1000
800
600
400
200
0
##
###
***
Vehicle
Prop 0.5 µg
Vehicle
Prop 0.5 µg
Control
IFS
REARING
Vetical activity (counts)
100
80
60
40
20
0
***
##
Vehicle
Prop 0.5 µg
Vehicle
Prop 0.5 µg
Control
IFS

Figure 5