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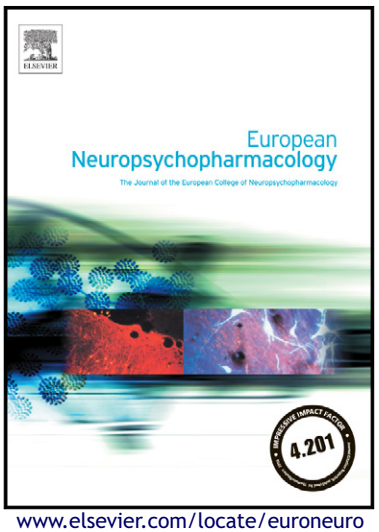

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Elevation of brain allopregnanolone rather than 5-HT release by short term, low dose fluoxetine treatment prevents the estrous cycle-linked increase in stress sensitivity in female rats

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Abstract

Withdrawal from long-term dosing with exogenous progesterone precipitates increased anxiety-linked changes in behavior in animal models due to the abrupt decrease in brain concentration of allopregnanolone (ALLO), a neuroactive metabolite of progesterone. We show that a withdrawal-like effect also occurs during the late diestrus phase (LD) of the natural ovarian cycle in rats, when plasma progesterone and ALLO are declining but estrogen secretion maintains a stable low level. This effect at LD was prevented by short-term treatment with low dose fluoxetine.

During LD, but not at other stages of the estrous cycle, exposure to anxiogenic stress induced by whole body vibration at 4Hz for 5min evoked a significant decrease in tail flick latency (stress-induced hyperalgesia) and a decrease in the number of Fos-positive neurons present in the periaqueductal gray (PAG). The threshold to evoke fear-like behaviors in response to electrical stimulation of the dorsal PAG was lower in the LD phase, indicating an increase in the intrinsic excitability of the PAG circuitry. All these effects were blocked by short-term administration of fluoxetine (2 x 1.75mg kg^{-1} i.p.) during LD. This dosage increased the whole brain concentration of ALLO, as determined using gas chromatography - mass spectrometry, but was without effect on the extracellular concentration of 5-HT in the dorsal PAG, as measured by microdialysis.

We suggest that fluoxetine-induced rise in brain ALLO concentration during LD offsets the sharp physiological decline, thus removing the trigger for the development of anxiogenic withdrawal effects.

Keywords

Estrous cycle, anxiety, periaqueductal grey matter, fluoxetine, allopregnanolone, *c-fos*

1. Introduction

Premenstrual syndrome (PMS) and its more severe counterpart: premenstrual dysphoric disorder, blights the lives of millions of women worldwide (Steiner, 1997). In susceptible women the symptoms, which include angry outbursts, irritability and anxiety (ACOG, 2001), can be considered an exaggerated and inappropriate response to everyday acute stressful challenges. Surprisingly, given the enormity of the problem of PMS, there has been relatively little basic scientific study into its neurophysiological basis.

What is clear from clinical studies is that PMS is dependent on cyclical variations in female sex hormones. Symptoms do not appear in anovulatory cycles (Backström et al., 2003). However ovulation itself is not the key factor since many women taking the combined contraceptive pill on a 21 day on, 7 day off dosing regimen, which prevents ovulation, also experience PMS-like symptoms, which peak during the 7 day drug free period (Kadian and O'Brien, 2012). In both cycling women and those taking the pill, symptoms occur at a time when blood levels of progesterone and estrogen, or their synthetic analogues, are in rapid decline.

In rats, withdrawal from long term dosing with exogenous progesterone at doses sufficient to raise progesterone to the high physiological range, precipitates increased anxiety (Smith et al., 1998; Smith et al., 2006). Spontaneously cycling rats in the late diestrus phase, when progesterone secretion declines sharply but estrogen secretion remains at a stable low level (Butcher et al., 1974), also become more fearful in an open field arena (Devall et al., 2009). Moreover, exposure of rats to 5min of mild anxiogenic vibration stress (5min vibration at 4Hz whilst confined in a tube, Jørum 1988) during late diestrus evokes a hyperalgesia, which is not seen when the animals are exposed to the same stressor at other stages of the cycle (Devall et al., 2009). These findings suggest that falling progesterone predisposes to an enhanced response to psychogenic stress. By analogy, the rapid decline in ovarian progesterone secretion in women during the late luteal phase might also provide a trigger for the enhanced responsiveness to psychogenic stress, which is a feature of the premenstrual period in many women (Nilni et al., 2011; Hoyer et al., 2013; Gollenberg et al., 2010). Thus whilst the cause of PMS in women is likely to be multifactorial, withdrawal from progesterone could be a significant contributory precipitating factor.

Progesterone passes readily through the blood brain barrier. The active agent that triggers the neuronal response to progesterone withdrawal in female rats is not however the native steroid hormone, but its neuroactive metabolite allopregnanolone (ALLO: 5α-pregnan-3α-ol-20-one or 3α,5α-tetrahydroprogesterone) (Gulinello et al., 2003; Smith et al., 1998). This progesterone metabolite ALLO is a potent positive allosteric modulator of the actions of GABA at $GABA_A$ receptors (Paul and Purdy, 1992)

and its concentration in random cyclic female rat brain correlates with that of its precursor progesterone in plasma (Corpéchot et al., 1993). Ovarian secretion of both progesterone and ALLO decreases sharply at late diestrus (Ichikawa et al., 1974; Holzbauer, 1975), and so the concentration of ALLO in the brain will decrease in parallel. There is some endogenous production of progesterone and ALLO in the male rat brain (Cook et al., 2014). A similar situation appears to exist in the female rat, as evidenced by the persistence of these steroids in the brains of ovariectomised and adrenalectomised animals, but this is at concentrations around 9-fold and 40-fold lower, respectively, than those seen in the brains of intact random cycling rats (Corpéchot et al., 1993). Even in female rats at late diestrus, when ovarian progesterone secretion is low, brain concentrations of ALLO and progesterone were 10-fold higher than in males (Fry et al., 2014). Whether naturally during the ovarian cycle or following administration of exogenous progesterone, withdrawal from ALLO, triggers upregulation of extrasynaptic $GABA_A$ receptors in the brain and consequent changes in excitability of neuronal circuits associated with anxiety (Gangisetty and Reddy, 2010; Griffiths and Lovick, 2005a; Griffiths and Lovick, 2005b; Gulinello et al., 2003; Lovick et al., 2005; Smith et al., 1998). The dynamic of the fall in brain concentration of progesterone appears critical because abrupt withdrawal from an exogenous progesterone-dosing regimen in rats precipitates an increase in responsiveness to anxiogenic stressors, whilst a gradually tapered withdrawal does not (Doornbos et al., 2009 ; Saavedra et al., 2006). In this respect it is interesting that in women, an association between clinical features of PMS and rate of decrease in progesterone during the luteal phase has been noted (Halbreich et al., 1986). Thus we reasoned that if the sharp fall in brain ALLO concentration that occurs at the end of the estrous cycle in rats is a trigger for the development of increased stress sensitivity, measures to produce a more gradual reduction in brain concentration of ALLO at the end of the estrous cycle should prevent the development of withdrawal-like symptoms.

In male rats and mice, the antidepressant fluoxetine (FLX) has been shown to induce an increase in the concentration of ALLO in the brain, which can be detected within 20 min of acute administration (Pinna et al., 2009; Serra et al., 2001; Uzunov et al., 1996). We therefore

hypothesized that if FLX produces a similar effect in female rats in late diestrus, then short term dosing with FLX during this stage of the ovarian cycle should offset the rapid physiological fall in the concentration of ALLO. Further, that the stimulus for the development of withdrawal effects, which normally characterise this phase of the ovarian cycle, should be absent and changes in behavioral responsiveness to stress should not occur. To test this hypothesis we chose to focus on changes in neuronal responsiveness in the PAG, since we have already shown that estrous cycle-linked changes in $GABA_A$ receptor expression and neuronal excitability occur in this structure alongside changes in behavioral responsiveness (Lovick et al., 2005; Devall et al., 2009; 2011). Moreover electrical stimulation of the PAG evokes behavior that characterises responses to threatening and hence stressful situations in the rat and is sensitive to actions of anxiolytic drugs (Borelli et al., 2004).

2. Experimental procedures

The following is a brief outline of the Methods used. Detailed descriptions are available as a Supplementary File.

2. 1 Animals and drug treatment regimen

The estrous cycle of female Wistar rats was established from daily vaginal smears taken at approximately 09.00h and only rats that had displayed at least two regular cycles used for the present study. Based on vaginal cytology, cycle stages were classified as follows: proestrus: mainly lymphocytes; estrus: mainly cornified cells; early diestrus: mainly leucocytes with well-defined lobular nuclei; late diestrus: fewer cells than in early diestrus, nucleus "clumped", presence of amorphous, disintegrating leucocytes (Brack and Lovick, 2007). Fluoxetine hydrochloride (FLX) (Sigma, 1.75 mg kg^{-1} i.p.) or saline vehicle was administered at 16:30-17:00 h on the evening of early diestrus. A second dose of fluoxetine or the saline vehicle was given in late diestrus the following morning 1h prior to behavioral or neurochemical testing. The animals

allocated for brain steroid measurement were killed by decapitation and the whole brain, minus the olfactory bulbs, removed rapidly and stored frozen at -80^{o}C until analysis. The dose of FLX was chosen as one that has been shown in male rats to produce a significant rise in brain concentration of ALLO (Uzunov et al., 1996) but is below the dose reported to produce significant rises in brain 5-HIAA and 5-HT levels (Fuller et al., 1974; Rutter and Auerbach, 1993) or to produce behaviorally measurable effects via actions on brain 5-HT systems after acute administration in vivo (≥10mg kg^{-1}) (Silva and Brandão, 2000).

2.2 Neurochemical studies

2.2.1 Brain allopregnanolone measurement

To establish whether the effects of FLX in preventing the behavioral and neuronal changes that normally occurred during late diestrus could be due to a steroidogenic action of the drug, we measured allopregnanolone concentration in whole brain homogenates. Rats were dosed with FLX (1.75mg kg^{-1} i.p.) or vehicle on the afternoon of early diestrus and again on the next morning when they were in late diestrus (see 2.1). In order to eliminate the possibility of eliciting stress-induced increases in allopregnanolone, this group of rats did not undergo behavioral testing. They were killed by decapitation 1h after receiving the final dose on the morning of late diestrus. The brain was removed rapidly, separated from the olfactory bulbs and the tissue immediately snap frozen in solid CO_2 pellets. Tissue was then stored at -80^{o}C prior to analysis of steroid content.

Following extraction and fractionation of free steroids, the concentration of allopregnanolone in brain homogenates was measured by selective ion monitoring of its methyloxime trimethylsilyl ether through gas chromatography - mass spectrometry (GC-MS), using the procedures described by Ebner and co-workers (Ebner et al., 2006) (for more details see Supplementary file).

2.2.2 Measurement of extracellular 5-HT concentration.

Guide cannulae were chronically implanted into the dPAG in 14 rats (see Supplementary file for details). Following a recovery period of 5 to 7 days, rats in the afternoon of their early diestrus phase were treated with FLX (1.75 or 10mg kg^{-1} i.p.) or saline following the same dosing protocol used for behavioral experiments (see above). Next day, in the morning of late diestrus, a microdialysis probe was inserted into the dPAG via the guide cannula. A stabilization period of 2h was allowed before commencing sample collection. First, samples of dialysate were collected every 30 min over a further 2h period to establish baseline values. The second injection of fluoxetine was then given and 6 more samples of dialysate were collected every 30 minutes. Thus the effect of FLX on concentration of 5-HT in the extracellular fluid in the dPAG was tested over the equivalent time period that behavioral testing was carried out in other groups of rats (see above). Since the dose of FLX that produced effects on behavior had no effect on extracellular 5-HT concentration in the PAG (see Results 3.1.2.2), as a positive control we also administered FLX at a higher dose of (10mg kg^{-1}) to another group of rats in late diestrus, in order to ensure that our system was able to detect changes in 5-HT concentration.

2.3 Behavioral studies

2.3.1 Induction of stress-induced hyperalgesia

Each rat was habituated, over three daily 30min sessions, to being constrained in a plexiglass tube in which it could rest comfortably but not turn around. Nociceptive threshold was assessed using the tail flick reflex in response to radiant heat applied to a 3mm diameter spot on the blackened underside of the tail. Tail flick latencies were measured at 5min intervals to establish a baseline (mean of 3 tail flick tests) before subjecting the rat to 5min of anxiogenic stress by vibrating the restraining tube at 4Hz for 5min. Tail flick testing then resumed for a further 20min (Devall et al.,

2009). Experiments were carried out on rats at all stages of the estrous cycle but each rat was tested only once, in order to avoid the possibility of learned effects due to repeated testing. Wherever possible, the experimenter was blinded to the estrous cycle stage of the animal. In all experiments, a video recording was made, so that behavior could be analysed off-line by other observers who were blinded to hormonal status.

2.3.2 Behavioral responses to direct electrical activation of the periaqueductal grey matter

Bipolar stimulating electrodes were chronically implanted into sites in the dorsal half of the periaqueductal gray matter (dPAG) (see Supplementary file for details). The PAG was stimulated for 10s periods (60Hz sine wave, intensity increased in 5μA increments) at pseudorandom intervals (30-120s) in order to determine the threshold current intensity needed to evoke the following behaviors (Brandão et al., 2008):

1. freezing (cessation of all movement apart from breathing, believed to represent evaluation of a distal threat),

2. escape (running and/or jumping, a measure of the response to proximal threat),

3. post-escape freezing (freezing that occurred after interruption of PAG stimulation at escape threshold).

The effect of PAG stimulation was tested on each rat at the four stages of its estrous cycle. The stage at which it was first tested was assigned randomly.

2.4 Functional activation of neurons in the PAG.

The effect of vibration stress on expression of the immediate early gene *c-fos* was examined in animals that had undergone behavioral testing for stress-induced hyperalgesia. Following the stress-testing protocol (see above) rats were returned to their home cages. Two hours following the exposure to vibration stress or at the equivalent time in non-stressed controls, they were

removed from their cages, anesthetized and the brain fixed by vascular perfusion, sectioned and processed to reveal Fos-like immunoreactivity. For each animal, the density of Fos-positive cells was sampled in 4 representative coronal sections through the PAG taken at antero-posterior levels -6.04, -7.04, -7.8 and -8.72 (Paxinos and Watson, 1986). For further details of the Method see Supplementary file.

2.5 Data analysis

The data was analysed using repeated measures two-way or one-way ANOVA, as appropriate with post hoc comparisons, or student's t-test as appropriate. Full details of statistical methods are available in the Supplementary Methods file.

3. Results

3.1 Neurochemical studies

3.1.1 Effect of fluoxetine on allopregnanolone concentration in late diestrus.

Allopregnanolone was detected in the brain tissue of all animals. However, in the FLX-treated group (n=5) the concentration was more than double that measured in the saline-treated group (n=6) (*P<0.01, unpaired t-test, Fig. 1A).

3.1.2. Effect of fluoxetine on extracellular brain 5-HT concentration in late diestrus.

Microdialysis samples for 5-HT measurement were taken after the second dose of FLX, i.e. at the equivalent time to when other rats were used for the behavioral testing or the collection of brain samples for ALLO measurement. One group of rats used for microdialysis received the same low doses of FLX (2 x 1.75 mg kg^{-1}) as animals used for the behavioral and neurochemical studies. In these rats that received the lower dose of FLX (1.75 mg kg^{-1}) the concentration of 5-HT taken in the period immediately prior to the second drug injection did not differ from the saline control

group (1.5± 0.15 pg μl^{-1} v. 2.4±0.42 pg μl^{-1} respectively). However, in the group that received the higher doses of FLX (2 x 10 mg kg^{-1}), the concentration of 5-HT prior to the second injection (3.8±1.07 pg μl^{-1}), was significantly higher than the saline-treated group ($p<0.05$, one-way ANOVA followed by Newman Keuls), presumably reflecting inhibition of serotonin re-uptake by this dose.

After the second injection of FLX, post-hoc comparisons showed that the lower dose of FLX (1.75mg kg^{-1}), i.e. the same dose as used in behavioral studies, still had no effect on the extracellular concentration of 5-HT in the dialysate compared to control group ($p > 0.05$) (Fig 1B). In contrast, the second higher dose of FLX (10mg kg^{-1}) caused a significant decrease in the concentration of 5-HT in the dialysate compared to baseline samples of the same group ($p < 0.05$) (Fig 1B). The minimum concentration of 5-HT (0.78±0.35 pg μl^{-1}) measured was above the lower limit of detection of our system (approximately 0.5 pg μl^{-1}). Histological analysis revealed that all the dialysis probes were localized to the dorsal PAG (Fig. 1C).

3.2. Behavioral testing

3.2.1 Effect of fluoxetine on the development of stress-induced hyperalgesia during late diestrus

Baseline tail flick latencies were similar at all stages of the estrous cycle and regardless of drug treatment status. In non-stressed controls and in saline-injected rats, tail flick latency did not change significantly over the course of the experiment (Fig 2). However, following exposure to 5 min vibration stress, the vehicle-treated animals in late diestrus, but not at other cycle stages, displayed hyperalgesia, manifested as a significant decrease in tail flick latency (TFL), lasting 20 min (Fig. 2). Administration of FLX (2 x 1.75mg kg^{-1} i.p) to rats in the late diestrus phase had no effect on baseline TFLs but blocked completely the development of stress-induced hyperalgesia (Fig 2).

3.2.2. Estrous cycle-linked changes in PAG-evoked fearful behavior - effect of fluoxetine.

In each rat the effect of electrical stimulation of the PAG was tested on 4 consecutive days i.e. at different stages of its estrous cycle. There was a significant estrous cycle-linked effect on the stimulus intensity required to evoke different aspects of fear-like behavior, as documented below.

Freezing and escape behaviors

Two-way ANOVA with repeated measures considering estrous cycle stage and treatments as the factors applied for freezing behavior showed significant differences between the stages of the estrous cycle but not between treatments. Also there was a significant interaction between the factors. *Post-hoc* comparisons indicated that the dPAG stimulation current intensity, which evoked freezing behavior, was significantly lower in late diestrus compared to other periods of the estrous cycle ($n=7$, $p < 0.05$) (Figs. 3A). Post-hoc comparisons for escape behavior determined that it was evoked at lower currents in late diestrus compared to proestrus and early diestrus (Fig. 3B). The analysis also clearly indicated that pre-treatment with FLX in late diestrus ($n=10$) prevented the estrous cycle-linked increase in sensitivity of the dPAG (Figs 3A and 3B). In contrast to the saline-treated rats, the threshold current required for eliciting freezing and escape behaviors in late diestrus in fluoxetine-treated rats did not differ from thresholds at other stages of the cycle.

Post-escape freezing

Two-way ANOVA with repeated measures applied on the duration of post-escape freezing behavior showed no difference between treatments or an interaction between treatments and stages (Fig. 3C).

Order of testing

In both the saline and FLX-treated group of animals, there was no correlation between thresholds for evoking fear-like behaviors and the cycle stage at which the first test was carried out. In the saline-treated rats the lowest thresholds always occurred during the late diestrus phase regardless of whether the rat was being tested for the first time or had been tested previously when in another stage of the estrous cycle. Similarly, there was no evidence for the development of tolerance to repeated testing over the 4 days of the experiment, regardless of the stage at which the rat was first tested ($p > 0.05$ in all cases). In all groups, the electrode placements were localized to the dorsal half of the PAG (dPAG) (Fig. 4).

3.3 Functional activation of neurons in the PAG.

We investigated expression of the immediate early gene *c-fos* as an index of functional activation of the PAG circuitry. In the animals that had undergone behavioral testing for stress-induced hyperalgesia (see above), Fos-like immunoreactivity was present in nuclei throughout the PAG. For the control rats (no vibration stress) the density of labelled cells was similar at all cycle stages (Fig. 5). And in rats that were exposed to 5min of vibration stress, the number of labelled cells present in the PAG in proestrus, estrus and early diestrus was not significantly different from the non- stressed rats. However, in rats in late diestrus, fewer labelled cells were present in comparison to other stages of the cycle, an effect which was most pronounced in the lateral part of the rostral half of the PAG (Fig. 5). This effect of vibration stress in LD was blocked completely by pre-treatment with FLX. Indeed, after FLX in late diestrus the number of Fos-immunoreactive cells in the PAG following vibration stress was increased significantly compared to saline-treated rats (Fig. 5). The effect of FLX was most pronounced in the lateral column of the rostral half of

the PAG where there was a threefold increase in the number of labelled cells compared to saline-treated controls (Fig. 5).

4. Discussion

In the present study, 5min exposure to anxiogenic vibration stress induced hyperalgesia during late diestrus but not at other stages of the cycle, as shown previously (Devall et al., 2009). Interestingly, during nociceptive flexor reflex threshold testing in women (arguably also a mildly stressful procedure) a mild hyperalgesia has been demonstrated during the luteal phase, similar to our finding in rats (Tassorelli et al., 2002). In addition, in the present study the thresholds for fear-like freezing and escape behavior evoked in response to direct electrical stimulation in the dPAG were found to be lower in late diestrus compared to other cycle stages. Changes in motor control seem an unlikely explanation for this effect since freezing and escape are opposing locomotor responses, yet both were sensitive in the same way to estrous cycle stage, i.e. a lowered threshold for evoking the responses in late diestrus. It also seems unlikely that the effect of PAG stimulation in late diestrus was pain related since it was blocked by fluoxetine, which is not known to have analgesic properties. Fluoxetine also prevented the development of vibration stress-evoked hyperalgesia in late diestrus as well as the reduction in the expression of Fos-like immunoreactivity in the PAG in response to the vibration stress.

The dose of fluoxetine chosen for this study (1.75mg Kg^{-1} i.p.) was at the lower end of the range reported to raise brain ALLO concentration in male rats (Uzunov et al., 1996), which was the only data available at the time our study began. Similar findings were subsequently reported in male mice by Pinna et al. (2009) and Serra et al. (2001), who showed that fluoxetine could raise brain ALLO concentration at doses below the threshold for effects on 5-HT systems. Indeed, the EC50s for an influence of fluoxetine on ALLO were 10-50 times lower than the EC50 required to inhibit 5-HT reuptake. Based on these findings it is possible that an even lower dose of fluoxetine might have been effective in the present study. Nevertheless, at the present dosage of FLX used to

prevent the stress-induced hyperalgesia of female rats in late diestrus, brain ALLO concentration was raised yet there was no change in the extracellular concentration of 5-HT in the dPAG. We cannot rule out an action of fluoxetine on 5-HT systems in other brain regions. However, the published data in males suggests that the dose we used would at best produce only small, transient effects (see Fig 1 of Rutter and Auerback, 1993). Moreover, fluoxetine has been shown to be less effective in raising extracellular 5-HT concentration in females compared to males after acute administration (Masswood et al, 1999). Thus it is most likely that the effects of fluoxetine in the present study were due its effects on neurosteroid synthesis rather than 5-HT systems.

In female rats, ALLO in the brain appears to arise predominantly from local metabolism of ovarian progesterone, with a significant correlation between brain ALLO and plasma progesterone across the estrous cycle (Corpechot et al., 1993). At late diestrus there is a sharp fall in plasma progesterone (Butcher et al., 1974) and the ovarian secretions of both this steroid and ALLO fall to the lowest values across the cycle (Ichikawa et al., 1974; Holzbauer, 1975). In male rats and in mice, a single dose (18 mg kg^{-1}) of fluoxetine produces a rapid onset (<15 min) increase in the brain concentration of ALLO, followed by a more gradual fall (> 2 h; Uzunov et al., 1996; Pinna and Rasmusson 2012). The elevation of brain ALLO by fluoxetine was thought to be due to an activation of the aldo-keto reductase enzyme, which produces this steroid from 5α-dihydroprogesterone (Griffin and Mellon 1999). Such a mechanism has been questioned however (Trauger et al., 2002) and we have shown recently that FLX raises ALLO concentration in female rat brain by inhibiting the microsomal dehydrogenase oxidising ALLO to 5α-DHPROG (Fry et al., 2014 in press). Acute treatment with FLX has been shown to inhibit the cytochrome CYP2C11 enzyme in liver (Wójcikowski et al., 2013) but this would not be expected to influence the synthesis of ALLO.

In our spontaneously cycling female rats, the short term dosage beginning late in the afternoon of early diestrus with a second dose on the morning of late diestrus, likely offset the

sharp decline in plasma and brain ALLO that normally occurs in late diestrus. However, administration of FLX did not simply shift the withdrawal effect on by a day since thresholds for evoking fear-related behaviors by electrical stimulation of the PAG a day later in proestrus were no different from saline-treated animals. The major metabolite of FLX is norfluoxetine, which Pinna et al. (2009) have shown to be more potent than FLX itself at elevating mouse brain ALLO and which in rat brain has a half-life of around 8h following a single dose (Qu et al., 2009). Thus it is likely that after administration of FLX in the current study, the brain concentration of ALLO was elevated throughout late diestrus.

Behavioral evidence for the estrous cycle-linked change in excitability of the PAG was accompanied by evidence of changes in the functional activation of neurons induced by exposure to mild anxiogenic vibration stress. Thus, in the early stages of the cycle (i.e. proestrus, estrus and early diestrus), exposure to the stressor failed to evoke a significant change in Fos expression in the PAG. This contrasted with late diestrus, when the vibration stress significantly lowered the number of Fos-positive neurons, particularly in the rostral half of the nucleus in the dorsal, dorsolateral and lateral columns. These results imply deactivation of a tonically active cell population in response to the acute stress applied during late diestrus. Reduced activation within GABAergic populations induced by stress is not unprecedented. In male rats, reduced activation of GABAergic neurons in the prefrontal cortex (i.e. fewer Fos-positive cells present) was reported following exposure to a novel anxiogenic stress (Weinberg et al., 2010) whilst a decrease in extracellular concentration of GABA has been observed in the PAG during contextual fear (Rea et al., 2009). Two recent studies in women also showed acute stress-evoked deactivation in the BOLD fMRI signal in both the PAG and the medial prefrontal cortex during the late follicular/mid-cycle (Goldstein et al., 2010) and the late luteal (Ossewaarde et al., 2010) phase of the menstrual cycle, the latter coinciding with increased sensitivity to acute emotional stress (Goldstein et al., 2010; Ossewaarde et al., 2010), as seen during the analogous ovarian cycle stage

in rats (see Introduction).

Although the phenotype/s of the Fos-labelled cells in the PAG in the present study was not identified, a stress-induced deactivation of the intrinsic GABAergic interneurone population during late diestrus would be consistent with the above findings in women. Afferent input from nociceptors is modulated at the spinal level by tonic activity in descending facilitatory and inhibitory systems that originate in the PAG (Gebhart, 2004). Under normal circumstances, the balance between the activity in these systems has been suggested to be biased towards facilitation, at least in males (Bee and Dickenson, 2008). Stress-induced inhibition of GABA tone on these control systems during late diestrus in females, might tip the balance further in favor of facilitation, thereby lowering the threshold for evoking nociceptive reflexes such as the tail flick. GABAergic neurones are present throughout the PAG of the rat and are especially populous in the dorsolateral sector (Griffiths et al., 2005a; Griffiths et al., 2005b; Lovick and Paul, 1999). In the cat they constitute some 36% of the total population in this sector (Barbaresi, 2005). In late diestrus or after withdrawal from dosing with exogenous progesterone, expression of α4, β1 and δ $GABA_A$ receptor subunits on the GABAergic interneuron population in the PAG is upregulated, triggered by the declining brain concentration of progesterone (and hence ALLO) (Griffiths and Lovick, 2005a; 2005b). Since α4, β1 and δ subunits can co-assemble to form functional receptors (Lovick et al., 2005) and the presence of δ subunits indicates an extrasynaptic location (Farrant and Nusser, 2005), the tonic current carried by the interneurone population of the PAG would increase. Indeed, we have shown *in vivo*, that the level of GABAergic tone, which regulates the excitability of the output neurons in the PAG, is reduced during late diestrus (Brack and Lovick, 2007). As a consequence, the circuitry becomes intrinsically more excitable, as reflected in the present study by the decrease at late diestrus in the threshold current required to evoke fear-like behaviors in response to electrical stimulation of the dPAG.

The above changes in behavior of the rats were associated with changes within the PAG at

the cellular level. Rather than simply normalizing Fos expression in late diestrus to the level seen in earlier stages of the cycle, FLX treatment transformed the stress-induced decrease in the number of fos-positive neurons in the PAG in late diestrus into a large increase in labelled cells, most prominently in the lateral column at mid-PAG level. Thus in the presence of FLX, the circuitry appeared to process the stress-inducing stimulus in a quite different way. This may reflect functional changes within the PAG circuitry itself and/or changes in afferent input from other structures involved in the processing of stress-inducing stimuli.

Fluoxetine was developed originally as a selective serotonin reuptake inhibitor and is one of the most widely used antidepressant drugs worldwide. Its clinical effectiveness as an antidepressant typically requires a long lead in period and exacerbation of adverse symptoms is not uncommon in the short term. In male rats, acute administration of FLX at doses that show anxiolytic or anti-aversive effects after long-term treatment is actually anxiogenic (Silva and Brandao, 2000). Yet in the present study of females in late diestrus, FLX appeared anxiolytic at the low dose (2 x 1.75mg kg^{-1}) used. Moreover, this low dose of FLX failed to produce a significant change in extracellular 5-HT concentration in the dPAG, suggesting that its effects on behavior were not due to an action on 5-HT systems.

The lack of effect of the lower dose of FLX (2 x 1.75mg kg^{-1}) used in the present study on extracellular 5-HT concentration in the PAG was not due to insufficient sensitivity of our system, because the basal concentration of 5-HT was above the threshold for detection. Moreover, a change in 5-HT concentration was observed in response to a higher dose of FLX (2 x 10mg kg^{-1}). Indeed the second higher dose of FLX, which we had used only to check the responsiveness of our microdialysis system, produced a significant decrease in the extracellular concentration of 5-HT in the dPAG. At first sight this finding appeared at odds with the reported rise in extracellular 5-HT in the PAG after acute administration of FLX in males (Zanoveli et al., 2010). However, in the dosing protocol used for our study in females the rats received FLX on two occasions: once on the

evening of early diestrus and again on the morning of late diestrus immediately before the microdialysis samples were taken. In the female rats that received the higher dose of FLX (10 mg kg^{-1}) the basal concentration of 5-HT before the second injection was given was significantly higher than in the control group. This suggests that the higher dose of FLX had blocked serotonin re-uptake in the PAG, as it does in males (Zanoveli et al., 2010). Against the raised basal level, a second injection of FLX might be expected to raise extracellular 5-HT further. However, a further rise in 5-HT in the dorsal raphe nucleus (DRN), the major source of serotonergic input to the PAG, may have lead to activation of 5-HT_{1A} somatodendritic autoreceptors, which would depress the activity of the DRN population (Rutter and Auerbach, 1993; Hajos et al., 2001) and reduce 5-HT output in the PAG, as seen in the present experiments. Whilst these possibilities are intriguing, further investigation was outside the scope of the present study.

ALLO has been shown to produce a modest increase the firing of 5HT neurons in the DRN after acute intracerebroventricular administration to anaesthetised rats (Robichaud and Debonnel, 2006). However, in that study the dose given of 1μg kg^{-1} icv would be expected, assuming an even distribution within the brain and without loss to the periphery, to give an ALLO concentration of about 0.15μg g^{-1} of brain for a 250-325g rat (Bailey et al., 2004). This is some 50 times higher than the ALLO concentration of 3ng g^{-1} brain measured after FLX treatment in the present study in females. Moreover, there was no change in the extracellular concentration of 5-HT in the PAG, which might have been expected if the FLX-induced rise in ALLO concentration had been sufficient to stimulate the 5-HT neurons in the DRN. Another possibility is that FLX could have influenced GABA release in the PAG or elsewhere via activation of the 5-HT_{2A} receptors that are present on GABAergic neurones in the PAG (Griffiths and Lovick, 2002). However, since our dose of FLX did not influence extracellular 5-HT concentration in the PAG, it is unlikely that these cells would have been influenced.

Our current findings in a rat model indicate that the increased responsiveness to acute

anxiogenic stress seen at late diestrus can be prevented by administration of FLX, which induces an increase in brain ALLO timed to offset the physiological decrease in the steroid expected at this stage of the ovarian cycle. The timing of FLX treatment appears to be critical, since dosing at other cycle stages had no effect on behavior. Moreover in a recent study using an exogenous progesterone withdrawal regimen, long-term treatment with FLX, albeit at a higher dose than that used in the present study, had no effect on the withdrawal response (Li et al., 2012). The pharmacological action of FLX might therefore be related to its ability to alter the rate of change of brain ALLO across the cycle rather than simply producing an increase in basal concentration. This would be consistent with our recent discovery that fluoxetine inhibits the oxidative inactivation of ALLO (Fry et al., 2014, in press).

In women, short term administration of FLX during the late luteal phase or after a single dose of a delayed release formulation, starting a few days before adverse premenstrual symptoms arise, could similarly produce a gradual tapering in brain concentration of this steroid over several days and thereby offset the precipitous fall in ALLO that occurs naturally. Fluoxetine is particularly suited to produce such an effect, with a long plasma half-life, which is seen also for its active metabolite norfluoxetine: 1- 4 and 7-15 days respectively after a single dose (Hiemke and Härtter, 2000). Moreover, if women at the late luteal phase are comparable to the late diestrus rats of the present study in requiring only a low dose of FLX to elevate brain ALLO concentration, then adverse side effects should be absent or minimal.

To conclude, short-term administration of a low dose of fluoxetine during late diestrus in the rat raises brain ALLO concentration and blunts the abrupt fall in this progesterone metabolite, which normally occurs at this stage of the ovarian cycle, due to the sudden decline in ovarian progesterone secretion. The trigger for the neuronal withdrawal response, which precipitates the development of anxiety-like behavior under stressful circumstances, is therefore absent. We suggest these observations in the rat provide an explanation for the efficacy of fluoxetine in the

treatment of PMS (see Majoribanks et al., 2013). If we are correct, then short term treatment with fluoxetine given at doses below the antidepressant threshold but tailored to elevate brain allopregnanolone during the premenstrual period, could explain both the rapid response of PMDD patients to fluoxetine (Steinberg et al., 2012) and the effectiveness of intermittent dosing with this drug in the treatment of the disorder (Steiner et al., 1997). Moreover, by determining the magnitude and rate of the decline in progesterone for each individual, it should be possible to personalise the treatment to select the minimum effective dose of fluoxetine for the patient.

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Figures captions

Fig. 1. A. Increase in brain concentration of ALLO (mean± S.E.M.) in rats in late diestrus treated with fluoxetine (FLX, 1.75mg kg^{-1} i.p., n=5) or saline vehicle (VEH, n=6) on the afternoon of early diestrus and again on the morning of late diestrus, (LD) 60 min before sacrifice. *P<0.01, unpaired t-test.

B. Extracellular concentration of 5-HT in the PAG of rats in late diestrus treated with fluoxetine (2 x 1.75 mg kg^{-1}, n=6 or 2 x 10 mg kg^{-1}, n=4 or saline, n=4 using the i.p. dosing regimen as above). Arrow indicates time of injection on morning of late diestrus. All values means ± S.E.M. *P<0.05 with respect to mean baseline, repeated measures two-way ANOVA with Tukey's test.

C. Location of dialysis probes (solid bars) plotted onto outlines of coronal sections through the PAG taken from the atlas of Paxinos and Watson (2007). Numbers below drawings indicate mm caudal to bregma.

Fig. 2. Effect of 5 min of vibration stress (gray bar) on tail flick latency (TFL) in female rats at different stages of the estrous cycle and following fluoxetine treatment. Administration of fluoxetine (FLX 1.75 mg kg^{-1} i.p.) or vehicle (saline, i.p.) was carried out on the evening of early diestrus and again 1 h before behavioral testing commenced in late diestrus the next morning. All values (mean ± SEM) are expressed as a percentage of mean pre-stress baseline values. Abbreviations: P: proestrus; E: estrus; ED: early diestrus; LD: late diestrus. * P<0.05, ** P<0.01, two-way ANOVA compared to baseline. § P<0.05, §§§ P<0.001, two-way ANOVA compared to vehicle (n=8 to10 per group).

Figure 3. Estrous cycle-linked effects on responses to electrical stimulation of the dPAG. Threshold currents to evoke freezing (A) and escape behavior (B). The duration of post-escape freezing is shown at C. For each rat thresholds were measured on the 4 days of its cycle. Saline (vehicle control, n=7) or fluoxetine (1.75mg Kg^{-1} *i.p,* n=10) was administered on the afternoon of early diestrus and again 1h prior to beginning testing on the morning of late diestrus.

Abbreviations P: proestrus; E: estrus; ED: early diestrus; LD: late diestrus; SAL: saline; FLX: fluoxetine. Data expressed as means ± S.E.M. * $p < 0.05$ compared to the other periods of the estrous cycle for freezing and to P and ED for escape and post-stimulation freezing behavior (Fischer´s LSD post-hoc test after significant repeated measures one-way ANOVA); # $p < 0.05$ significant interaction between treatments and stage of estrous cycle.

Fig 4. Left: Photomicrograph showing location of electrode track in the dorsal PAG in a representative animal. Right: Stimulation sites in the dorsal PAG plotted onto representative outline sections of the PAG taken from the atlas of Paxinos and Watson (2007). o: no drug (n=12); *: saline-treated rats (n = 7); ■: fluoxetine-treated rats (n = 10). Numbers below sections indicate distance from bregma.

Fig. 5. A. The density of Fos-positive nuclei in 5 longitudinal columns in the rostral (levels I & II) and caudal (levels III & IV) halves of the PAG. Panel B shows the effect of vibration stress and fluoxetine (1.75 mg kg^{-1} i.p.) treatment in late diestrus on the density of Fos-positive nuclei in whole extent of the PAG observed at each stage of the estrous cycle. Panel C shows detailed effect of FLX in different columns of the rostral (levels I and II) and caudal (levels III and IV) PAG in late diestrus. All values mean ± SEM, n=5-6 per group. Abbreviations: P: proestrus; E: estrus; ED: early diestrus; LD: late diestrus. *** P<0.001, ** P<0.01, * P<0.05, § significantly different from stressed animals in proestrus, estrus and early diestrus (P<0.05), two-way ANOVA with Bonferroni's test.

Conflict of interest
The authors declare no conflict of interest

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Author Contributors
The bulk of the experimental work was carried out by Adam Devall
and Julia Santos who also analysed data. Jonathan Fry developed the protocol for steroid measurement with input from John Honour, carried out some of the experimental work and analysed all the data. Marcus Brandão advised on the work carried out in Brazil. Thelma Lovick conceived the study, contributed to experimental work and analysis of data and wrote the draft of the paper, with input from Jonathan Fry. All authors approved the final version of the paper.

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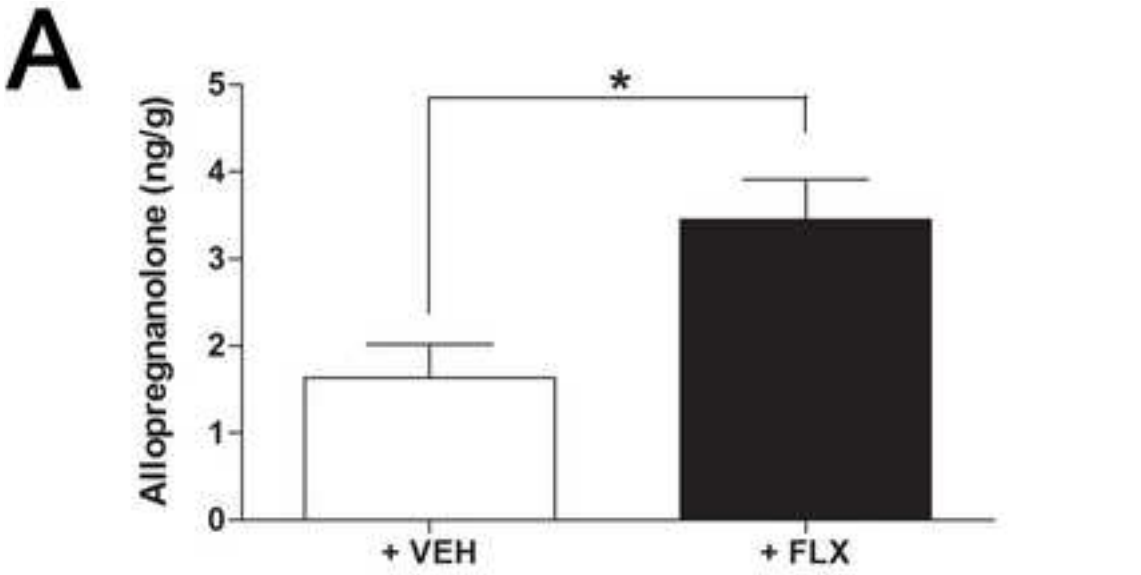

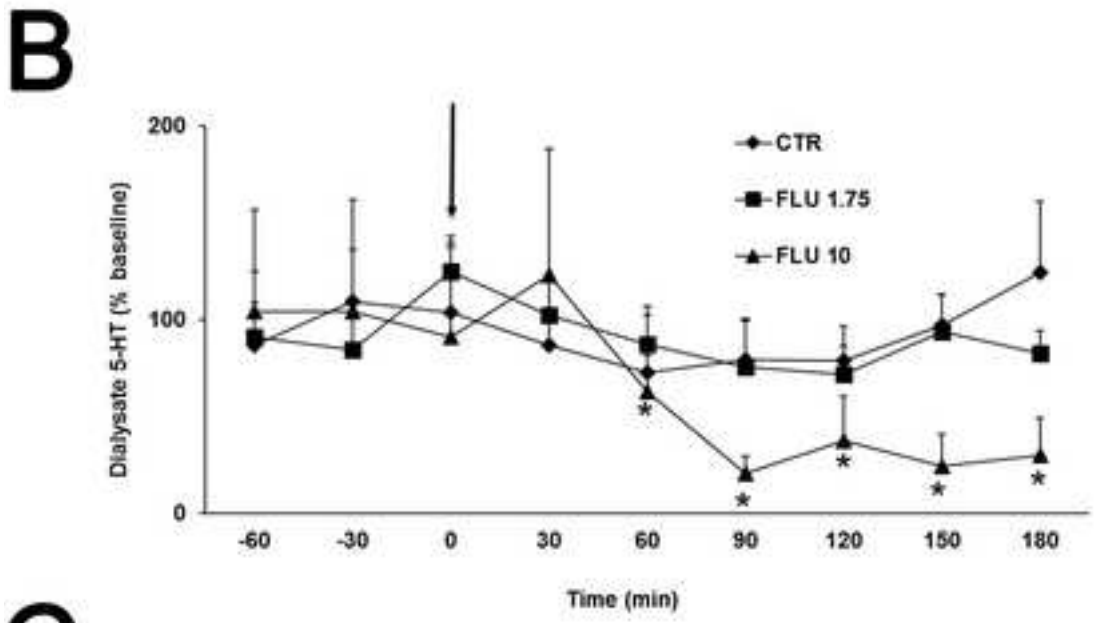

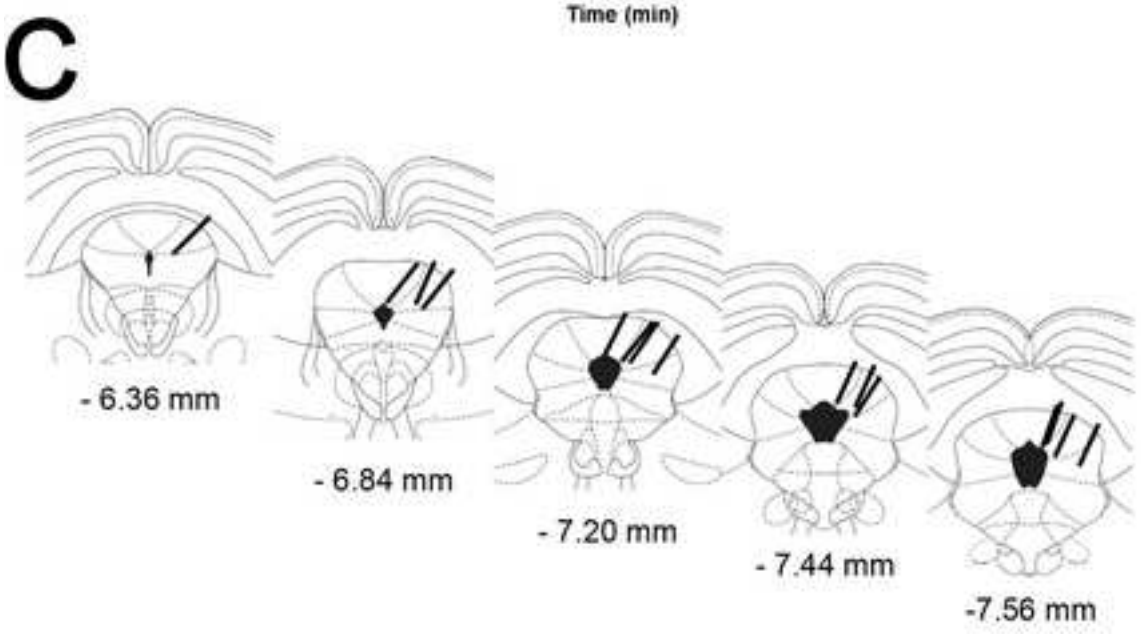

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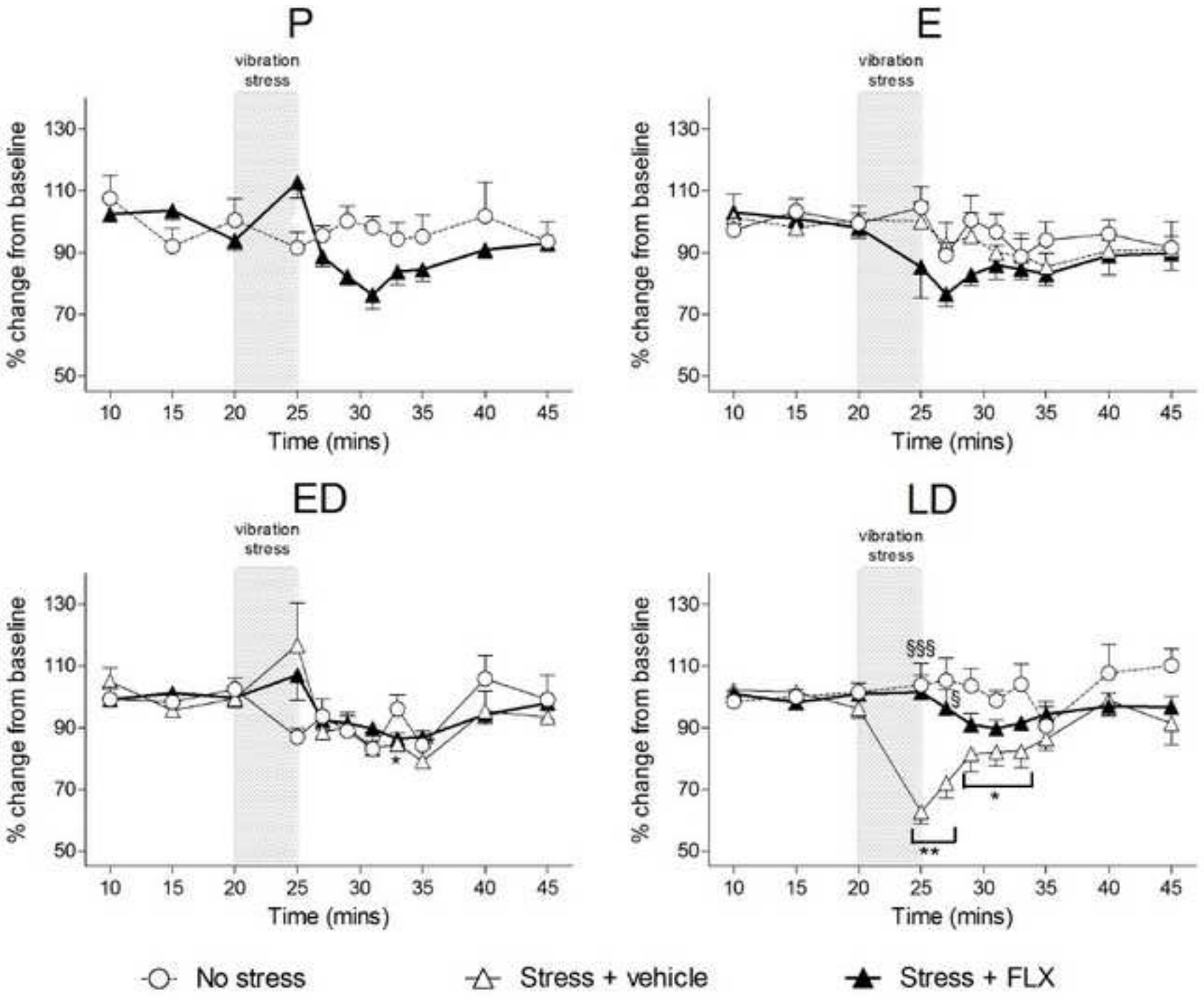

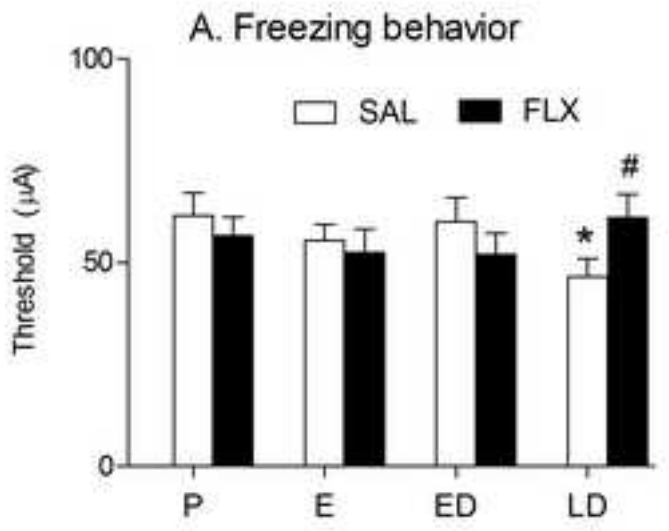

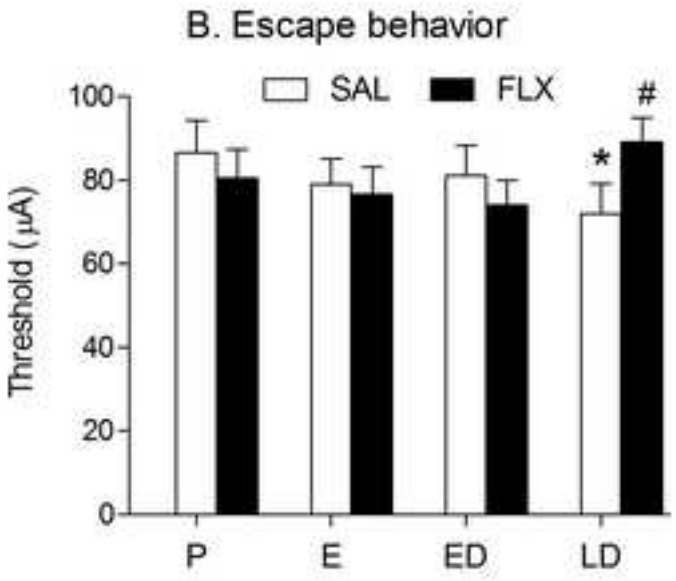

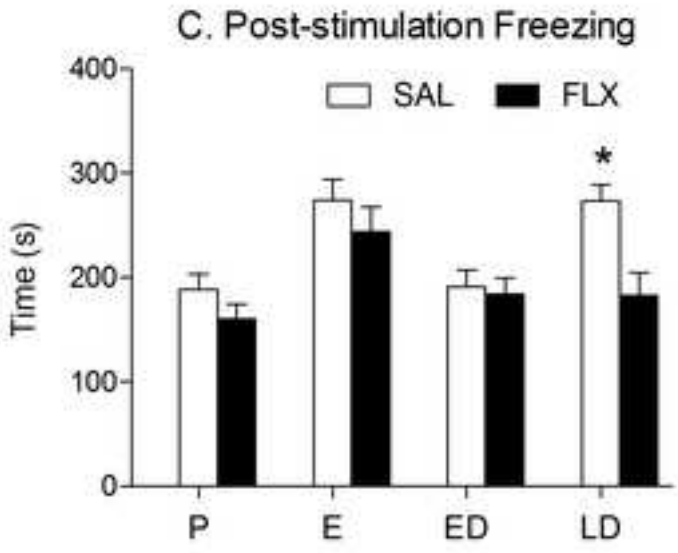

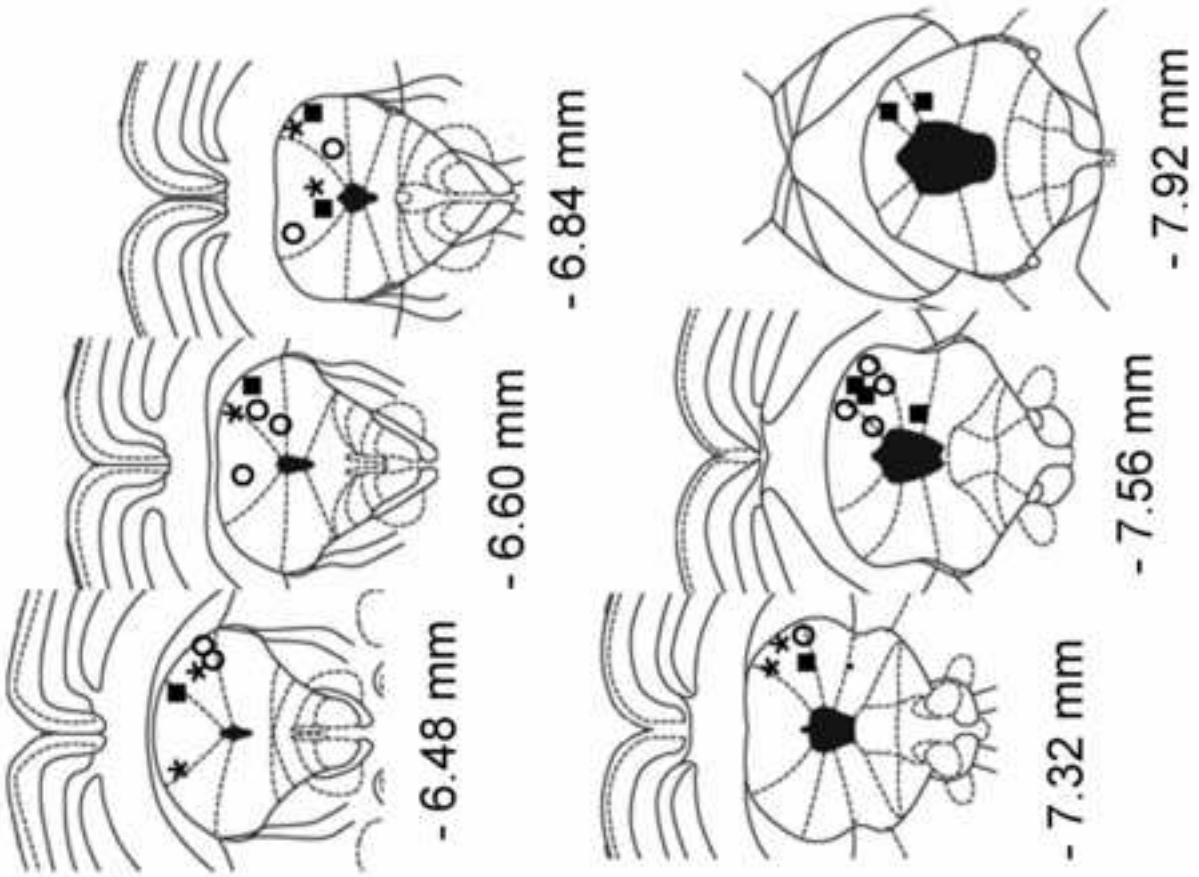

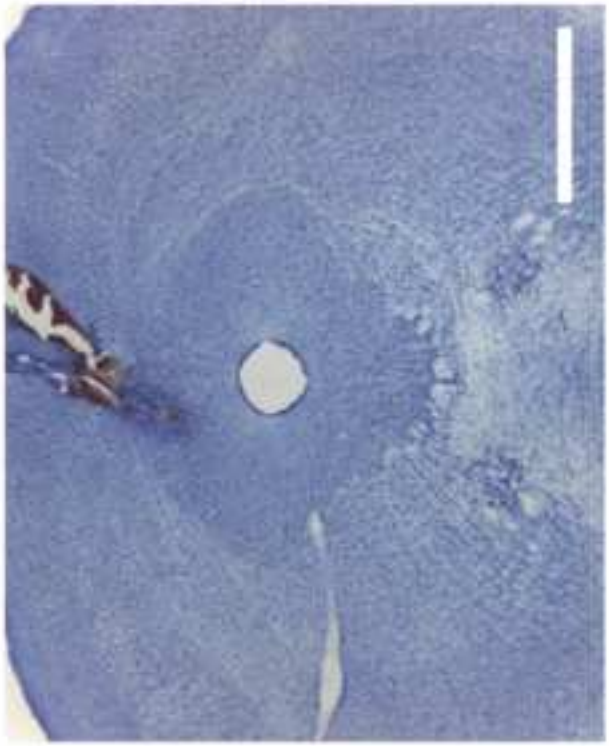

Figure(s)

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