Citalopram-mediated anxiolysis and differing neurobiological responses in both sexes of a genetic model of depression.

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Abstract

Disorders such as depression and anxiety exhibit strong sex differences in their prevalence and incidence, with women also differing from men in their response to antidepressants. Furthermore, receptors for corticotrophin releasing hormone (CRHR1) and arginine vasopressin (AVPR1b) are known to contribute to the regulation of mood and anxiety. In the present study, we compared the anxiety profile and CRHR1 and AVPR1b expression levels in control Sprague Dawley (SD) rats and rats of the SD-derived Flinders Sensitive Line (FSL), a genetic model of depression. Additionally, given the apparent sex differences in the therapeutic efficacy of antidepressants and because antidepressants are commonly used to treat comorbid anxiety and depressive symptoms, we assessed whether the anxiolytic effects of an antidepressant occur in a sex-dependent manner. Male and female FSL rats were treated with citalopram 10mg/kg once daily for 14 days and were then tested in the open field and the elevated plus maze paradigms. Upon completion of the behavioural analysis, AVPR1b and CRHR1 expression levels were monitored in the hypothalamus and the prefrontal cortex (PFC) using western blotting. According to our results, male FSL rats were more anxious than control SD rats, a difference abolished by citalopram treatment. Baseline anxiety levels were similar in female FSL and SD rats, and citalopram further reduced anxiety in female FSL rats. Importantly, whereas citalopram altered AVPR1b expression in the hypothalamus of male FSL rats, its actions on this parameter were restricted to the PFC in female FSL rats. In both sexes of FSL rats, citalopram did not alter CRHR1 expression in either the hypothalamus or PFC. Our results demonstrate that antidepressant treatment reduces anxiety levels in FSL rats of both sexes: the magnitude of treatment effect was related to the starting baseline level of anxiety and the antidepressant elicited sexually differentiated neurobiological responses in specific brain regions.

Keywords

Antidepressant; Sex differences; Anxiety; Depression; Females; Receptors

Abbreviations

ANCOVA: Analysis of Co-Variance

AVP: Arginine Vasopressin

AVPR1b: Arginine Vasopressin Receptor subtype 1b

CRH: Corticotropin Releasing Hormone

CRHR1: Corticotropin Releasing Hormone Receptor subtype 1

EPM: Elevated Plus Maze

FSL: Flinders Sensitive Line

HPA: Hypothalamic Pituitary Adrenal

SD: Sprague Dawley

SSRI: Selective Serotonin Reuptake Inhibitor

Article Highlights

- Male, but not female, Flinders Sensitive Line of rats present anxiety behaviour
- Repeated citalopram treatment reduces anxiety levels in both sexes
- Citalopram modulates AVPR1b expression in a sex-specific manner
- Differing mechanisms converge to produce anxiolysis in FSL rats of both sexes

1. Introduction

Stress and dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis are implicated in the pathophysiology of mood and anxiety disorders (Tsigos and Chrousos, 2002), which show greater comorbidity and prevalence in women (Kessler et al., 1994, Alonso et al., 2004). Two hypothalamic drivers of the HPA axis, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), also influence mood and emotional behaviour through their actions at extra-hypothalamic sites (Holsboer and Ising, 2008). The actions of CRH are mediated by two receptor subtypes (Lowry and Moore, 2006). The CRH subtype 1 receptor (CRHR1) modulates context-dependent affective responses and is expressed in the hypothalamus and extra-hypothalamic areas, including the frontal cortex (Sanchez et al., 1999). On the other hand, cerebral vasopressin pathways show distinct anatomical and functional patterns (Ermisch et al., 1993); most central actions of this peptide are mediated by subtype 1b vasopressin receptors (AVPR1b) which are also expressed in the hypothalamus and frontal cortex (Hernando et al., 2001). Increasingly, AVPR1b are being implicated in behaviours relevant to mood and anxiety disorders (Surget and Belzung, 2008). Antagonism of either CRHR1 or AVPR1b results in anxiolytic and antidepressant effects, suggesting these receptors as alternatives to the current monoaminergic drug targets (Surget and Belzung, 2008, Binder and Nemeroff, 2010).

Sex differences in the activity of the HPA axis are well known and the sexually differentiated response to stress is increasingly thought to be an important underlying factor in mood and anxiety disorders (Young, 1998, Goel and Bale, 2009, Young and Korszun, 2010). Women are nearly twice as likely to suffer from depression (Kessler et al., 1994, Alonso et al., 2004). Moreover, as compared to men, depressed women make a greater number of suicide attempts, show more somatisation, anger and hostility, and display increased appetite and weight gain. Importantly, although melancholic depression occurs equally in both sexes, the anxious and atypical forms

of depression are more commonly found in women (Frank et al., 1988, Marcus et al., 2005, Marcus et al., 2008), although the construct of anxious depression has received some criticism (Nelson, 2008). It is now well-established that estrogens influence depressive symptoms, including irritability, insomnia, appetite, and general physical well-being (Young et al., 2007, Kornstein et al., 2010). Together, these observations suggest the potential importance of considering the role of sex and the ovarian steroid milieu in measuring the efficacy of antidepressant therapy. Indeed, several studies have reported that women respond better to selective serotonin re-uptake inhibitors (SSRI) (Kornstein et al., 2000, Joyce et al., 2003, Baca et al., 2004, Khan et al., 2005); a similar conclusion was reached in a recent large multicentre trial of citalopram (Marcus et al., 2008). Nevertheless, some authors have argued that sex differences in response to antidepressant treatment may be clinically irrelevant (Quitkin et al., 2002, Hildebrandt et al., 2003, Parker et al., 2003, Wohlfarth et al., 2004, Thiels et al., 2005).

Experimental studies from our group and others have consistently demonstrated increased female vulnerability to the detrimental effects of stress on mood- and anxiety-related behaviours (Patchev and Almeida, 1998, Dalla et al., 2010, Pitychoutis and Papadopoulou-Daifoti, 2010, Pitychoutis et al., 2010). Furthermore, our previous studies showed that antidepressants elicit similar, albeit differing in magnitude, behavioural responses in male and female rats of the Flinders Sensitive Line (FSL); serotonergic and glutamatergic responses also differ between the two sexes following antidepressant treatment (Kokras et al., 2009a, Kokras et al., 2009b). FSL rats, derived by selective breeding of Sprague-Dawley (SD) rats, have several key characteristics which support the face, construct and predictive validity of this model of depression (Overstreet, 2002). FSL rats exhibit decreased weight and disturbed appetite, have elevated REM sleep, present anhedonia in chronic mild stress and are less active in novel environments; at the same time they are inherently more immobile in the forced swim test. Furthermore, in brain limbic areas, FSL rats

show significant regional abnormalities in levels of biogenic amines and their receptors, reduced serotonin synthesis, impaired serotonin-induced dopamine release, and impairments in their immune system (Overstreet et al., 2005). Importantly, chronic and not acute treatment with antidepressants restores those observed behavioural and neurobiological abnormalities (Yadid et al., 2000).

Despite the large body of evidence supporting the validity of the FSL model of depression, it still remains unclear whether FSL rats can model comorbid anxious depression. Previous research on male FSL showed increased levels of anxiety in the social interaction test (Overstreet, 2002, Janowsky et al., 2004, Lavi-Avnon et al., 2005) but not in the elevated plus maze (EPM) (Schiller et al., 1991, Overstreet et al., 1995). Whereas male FSL rats have been well studied, relatively fewer studies have focused on female FSL animals. While our previous studies confirmed the validity of female FSL rats as a model of depression (Kokras et al., 2009a, Kokras et al., 2009b), there is no information with respect to their anxiety profile and expression levels of CRHR1 and AVPR1b.

Based on the aforementioned sex-differences and the documented validity of male and female FSL rats as a model of depression, a first aim of the present study was to explore the potential suitability of the FSL rat as a model of comorbid anxious depression. Further, given that male FSL rats were previously reported to be sensitive to the behavioural actions of CRHR1 and AVPR1b antagonists (Janowsky et al., 2004, Overstreet et al., 2004, Overstreet et al., 2005) we examined the expression of brain CRHR1 and AVPR1b in male and female FSL rats under baseline conditions and after repeated citalopram treatment.

2. Experimental Procedures

2.1 Animals

Twenty-four adult male and female FSL rats, weighing 275±17 g and 200±15 g, respectively, and aged 10-11 weeks at the beginning of the experiment, were used. In addition, 24 male and female, similarly aged, Sprague-Dawley rats, weighing 325±26 g and 245±20 g respectively, were used as controls as previously described (Overstreet et al., 2005). Animals were group-housed, according to sex, under controlled 12:12 light/dark cycles (lights on at 07:00 a.m.) and temperature (22±2 °C), with free access to food and tap water. All animal experiments were carried out in accordance with the EEC directive 86/609.

2.2 Oestrous Cycle

In the case of females, a semi-random process controlled for disparities regarding the phases of the oestrous cycle. Specifically, female rats were selected from a larger pool of experimental animals on the basis of a normal 4-5 day cycle and assigned to groups on the basis of an equal distribution of oestrous cycle phases; the latter was monitored by vaginal smears until the day of sacrifice, as described elsewhere (Becker et al., 2005). Oestrous cycle phases on the day of behavioural testing and sacrifice are reported in Table 1.

2.3 Treatments

FSL and control SD rats were gently handled, daily by the same researcher. Male and female animals were given intra-peritoneal injections of either saline (FSL n=12; SD n=12) or 10mg/Kg of citalopram (Lundbeck S.A., Denmark; FSL n=12; SD n=12) during the morning, over 14 days. This dose of citalopram was previously shown to be effective and has been routinely used in male and female rats (Burghardt et al., 2004, Overstreet et al., 2004, Hasegawa et al., 2005).

2.4 Open Field

Spontaneous activity under novelty stress was measured for 5 min, approximately 22-26 h after the last injection of saline or citalopram (day 15). As previously described (Pitychoutis et al., 2009b), all rats were acclimatized to the test room for 1 h and thereafter placed in a clear Plexiglas chamber (Med Associates Inc., St Albans, VT) measuring 430 x 430 x 300 mm with arrays of 16 x 16 photodetectors, positioned 2.5 cm and 10 cm above the floor of the chamber. Interruption of adjacent photobeams provided an index of ambulatory activity (horizontal activity) while interruption of the upper line of photobeams provided an index of rearing behaviour (vertical activity). The time spent at the centre of the chamber served as an index of anxiety (Belzung and Griebel, 2001).

2.5 Elevated Plus Maze

The Elevated Plus Maze (EPM) test was conducted under dim light, approximately 1 min after the open field test. The test sequence from the open field to the elevated plus maze is known to enhance exploratory activity and to improve the reliability of plus-maze testing (Pellow et al., 1985). Each rat was placed facing an open-arm in the middle of the EPM, which was placed 50 cm above the ground and consisted of two open (50 x 10 cm) and two identical arms, enclosed within 50 cm high walls with a symmetrical 100 cm^2 square area at the centre. The number of total and open arm entries was recorded for 5 minutes. An index of anxiety was obtained from the ratio of the time spent in the open arms vs. the total time in the maze (Doremus et al., 2006). Upon completion of the behavioural testing, rats were returned to their home cages.

2.6 Western blot analysis

Twenty minutes after behavioural analysis, all animals were rapidly sacrificed and the prefrontal cortex and the hypothalamus were dissected out, snap-frozen in liquid nitrogen and stored at –80° C. Their brains were rapidly removed and for the

dissection of the regions of interest a method described in detail by (Heffner et al., 1980) was followed with minor modifications, as described in (Kokras et al., 2009b). In short, two sagittal sections, rostrally to caudally, separated the prefrontal cortex from the frontal lobe and the rest of the brain. Samples were homogenized using a Dounce glass homogenizer in lysis buffer containing 100 mM Tris-HCl, 250 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 1% NP-40, Complete Protease Inhibitor (Roche, Mannheim, Germany) and Phosphatase Inhibitor Cocktails I and II (Sigma, St. Louis, MO). Extracts were cleared by centrifugation (14000 g) and protein contents were estimated by Lowry assay. Protein lysates (40ug) were electrophoresed on 10% acrylamide gels in Laemmli buffer (250 mm Tris-HCl, pH 6.8, containing 4% SDS, 10% glycerol, 2% β-mercaptoethanol, and 0.002% bromophenol blue) and transferred onto nitrocellulose membranes (Protran BA 85, Schleicher & Schuell, Dassel, Germany). The membranes were blocked in TBS containing 5% non-fat milk and 0.2% Tween-20 before incubation with anti-AVPR1b (1:1000; AB3510P, Chemicon, Temecula, CA) and anti-CRHR1 kindly provided by Dr. E.A. Linton (1:200; Oxford), described in (Castro et al., 1996). For normalization purposes, blots were also probed with anti-β-actin (1:2000; Chemicon). Following incubation with horseradish peroxidase–IgG conjugates (Amersham Biosciences, Freiburg, Germany), antigens were revealed by enhanced chemiluminescence (ECL, Amersham Biosciences). Band intensities were evaluated by densitometry and all values were normalized and expressed as percentages of control male vehicle – treated SD rats.

2.7 Corticosterone assay

Trunk blood samples were collected at sacrifice and processed to recover serum (centrifugation at 4000 *g*, 30 min, 4°C); serum samples were stored at -20° C before being assayed for corticosterone by a standard radioimmunoassay (MP

Biomedicals, Costa Mesa, CA). The inter- and intra-assay coefficients of variation were both 8%.

2.8 Statistical Analysis

All results presented herein were analyzed with three-way ANCOVA using the General Linear Model of SPSS version 19 (IBM Corp, Somers, NY, USA) with "strain" (FSL vs. control SD), "sex" (male vs. female) and "drug" (citalopram vs. vehicle) as independent variables. Subsequent one-way ANCOVAs were performed to elucidate significant results as indicated by the factorial model. Specifically for corticosterone, pearson's correlations with behavioural outcomes were calculated. Oestrous cycle phase was used as a covariate in all factorial analysis to control variance due to the female hormonal milieu and to increase the validity of the male/female comparison. In this study, the main effect of oestrous cycle was not significant in any of the statistical analyses described further on; however this study was not designed to specifically detect oestrous cycle effects but merely to control for them. A probability value of $p \leq 0.05$ was considered as significant.

3. Results

3.1 Behavioural measurements

3.1.1 Open Field test

Horizontal activity. Statistical analysis indicated a significant "sex" main effect, as female FSL and SD rats generally showed higher ambulatory counts than their strain-matched male controls [$F_{(1,39)}=27.553$ $p<0.001$]. Furthermore, a significant "strain" main effect was observed [$F_{(1,39)}=9.259$ $p=0.004$], reflecting the lower ambulatory counts of FSL rats during the 5 min open field test. Male FSL rats

displayed considerable lower horizontal activity than their SD counterparts [$F_{(1,10)}$=46,507 p<0.001] (Figure 1a).

Vertical Activity. Statistical analysis revealed a significant "sex" main effect [$F_{(1,39)}$=38.263 p<0.001], as female SD and FSL rats generally had higher vertical counts than males of the corresponding strains (Figure 1b).

Time in centre. The factorial model revealed significant "strain x sex" and "strain x drug" interactions [$F_{(1,39)}$=8.344 p=0.006; $F_{(1,39)}$=10.429 p=0.003]. Further analysis showed that male, but not female, FSL rats spent less time in the centre of the arena than their sex-matched SD counterparts [$F_{(1,10)}$=5.171 p=0.046]. Citalopram-treated male FSL rats displayed considerably more time in the centre of the arena than vehicle-treated male FSL rats [$F_{(1,10)}$=27.128 p<0.001]. Interestingly, citalopram-treated female FSL rats also showed a significant increase in the time spent in the centre of the arena [$F_{(1,9)}$=8.749 p=0.016] (Figure 1c).

3.1.2 Elevated Plus Maze test

Total arm entries. Statistical analysis revealed a significant "sex" main effect [$F_{(1,39)}$=4.288 p=0.045], indicating that female SD and FSL rats made more arm entries than SD and FSL males, respectively (Figure 2a).

Open arm entries. Regarding open arm entries, a significant "sex" main effect [$F_{(1,39)}$=6.443 p=0.015] was found, indicating that female SD and FSL rats make more open arm entries than their male counterparts (Figure 2b).

Time in open arms. Statistical analysis uncovered significant "strain x sex" and "strain x drug" interactions on this parameter [$F_{(1,39)}$=8.305 p=0.006;

$F_{(1,39)}$=15.002 p<0.001 respectively]. As compared to respective SD controls, male, but not female, FSL rats spent less time in the open arms of the apparatus [$F_{(1,10)}$=126.772 p<0.001]. However, citalopram treatment elongated the time spent in the open arms in FSL rats of both sexes [males: $F_{(1,10)}$=24.874 p=0.001; females: $F_{(1,9)}$=5.666 p=0.041] (Figure 2c).

3.3 Neurobiological measurements

3.3.1 Corticosterone Serum Levels

Statistical analysis revealed that female FSL and SD rats had significantly higher corticosterone serum levels [$F_{(1,39)}$=20.314 p<0.001]. Furthermore, male FSL rats had significantly higher corticosterone levels than control male SD rats [$F_{(1,10)}$=35,426 p<0.001]. Citalopram treatment mediated a significant decrease in corticosterone levels in male FSL rats as compared to vehicle treated male FSL rats [$F_{(1,10)}$=17.380 p=0.002]. However, citalopram treatment had no effect in female FSL rats (Figure 3a). Furthermore, Pearson's correlation analysis showed that corticosterone levels significantly and inversely correlated in males only with the time spent in the centre of the open field and in the open arms of the EPM [$r_{(24)}$= -0.590, p=0.002; $r_{(24)}$= -656, p<0.001 respectively], as shown in the scatter plot (figure 3b). These correlations were not significant for female rats. Similarly, a linear regression model for corticosterone with behavioural and neurobiological measurements as predictors did not produce significant results.

3.3.2. CRHR1 expression levels

Immunoblotting was used to monitor CRHR1 levels in the hypothalamus and PFC. Regarding hypothalamic CRHR1, the analysis revealed a significant "strain" main effect; male and female FSL rats were found to have significantly lower levels of CRHR1 expression than SD rats [$F_{(1,39)}$=11.478 p=0.002] (Figure 4a). Citalopram

exhibited no effect on CRHR1 levels. A similar analysis of CRHR1 expression in the PFC indicated a significant "strain x drug" interaction [$F_{(1,39)}$=6.617 p=0.014] (Figure 4b). Further analysis showed that, similar to observations in the hypothalamus, the prefrontal cortex of male and female FSL rats exhibits lower levels of CRHR1 than their SD counterparts [$F_{(1,39)}$=50.330 p<0.001]. Notably, citalopram treatment resulted in differential effects in the two rat lines: while the antidepressant failed to produce any effect in male and female FSL rats, it increased CRHR1 expression in male and female SD rats [$F_{(1,10)}$=7.024 p=0.024; $F_{(1,9)}$=5.565 p=0.043] (Figure 4b).

3.3.3. AVPR1b expression levels

The statistical analysis for AVPR1b hypothalamic expression levels indicated a significant "strain x sex x drug interaction" [$F_{(1,39)}$=9.067 p=0.005] (Figure 4c). Vehicle-treated male, but not female, FSL rats showed higher hypothalamic AVPR1b expression levels than their SD counterparts [$F_{(1,10)}$=24.238 p=0.001]. Further analysis revealed that citalopram lowered hypothalamic AVPR1b expression levels in male [$F_{(1,10)}$=8.762 p=0.014], but not female, FSL rats (Figure 4c). Regarding cortical AVPR1b expression levels, the analysis showed a significant "sex x drug" interaction [$F_{(1,39)}$=7.163 p=0.011]. Further analysis showed that female FSL rats had lower cortical AVPR1b than the sex-matched SD rats [$F_{(1,9)}$=11.440 p=0.008]. Finally, whereas citalopram did not have a significant effect in males, it robustly increased cortical AVPR1b in female FSL rats [$F_{(1,9)}$=5.674 p=0.041] (figure 4d).

4. Discussion

Although depression and anxiety have greater prevalence and different phenomenology in women, the biological mechanisms underlying such sex differences are not well understood. Likewise, reports on the sex-differentiated antidepressant response remain controversial. The present study used a well-validated model of depression and explored its suitability as a model of comorbid

anxious depression, taking into consideration sex-differences in anxiety and depression at baseline and following treatment. In addition, this study explored possible links between anxiety-like behaviour, corticosterone levels and the expression of CRHR1 and AVPR1b in FSL animals under baseline conditions and post-antidepressant treatment.

Our results showed that male, but not female, FSL rats are less active and show greater signs of anxiety than their sex-matched SD controls. These findings are in agreement with the previously reported anxious profile of male FSL rats in the social interaction test (Overstreet, 2002, Janowsky et al., 2004, Lavi-Avnon et al., 2005). Differences between the present EPM results and those reported previously (Schiller et al., 1991, Overstreet et al., 1995) may be explained by methodological differences. In fact, male FSL rats adopt passive coping strategies when challenged in behavioural tests (Overstreet, 2002, Overstreet et al., 2005) and the sequential behavioural testing along with the repeated injections in this study, possibly provoked the anxiety-like behavioural response of male FSL rats.

In contrast to males, female rats (of both strains) were generally more active, as reported by others too (Brotto et al., 2000, Romero and Chen, 2004). This is thought to reflect sex-specific coping strategies and hormonal environment (Palanza, 2001, Dalla et al., 2010); the latter is particularly relevant since gonadal hormones are known to influence activity in rodents (Nomikos and Spyraki, 1988, Becker et al., 2005) and phenomenology of depression in humans (Kornstein et al., 2010). Rather than focussing on the contribution of individual gonadal hormones using ablation and hormone replacement strategies (Becker et al., 2005), the present experiments were designed to average and control for such potentially confounding effects. Thus, while we previously confirmed a depressive-like phenotype in female FSL rats (Kokras et al., 2009a), it was interesting to find here that FSL females do not express anxiety-like behaviour. This finding is in contrast to those reported in humans, given that

previous research has established that women present more often anxious depression than men (Frank et al., 1988, Marcus et al., 2005, Marcus et al., 2008).

Based on the aforementioned observations, we conclude that while the FSL rat is a well-validated genetic model for studying depressive-like behaviour, anxiety in male FSL rats is dependent on the experimental protocol, hence the previously conflicting results. In addition, while female rats were not anxious in our study, we cannot exclude that using other protocols they might also present anxiety. In summary, our results point towards anxiety being state, rather than trait, dependent in FSL rats; this limits their potential as a model of anxious depression. Interestingly, should we have used only male FSL rats in our study, the unexpected sex-differentiated anxious profile of FSL rats, being the polar opposite of the human condition, would have gone unnoticed. Therefore, in agreement with previous suggestions (Palanza, 2001, Dalla et al., 2010), our findings highlight the importance of considering both sexes when studying animal models of disease.

With regards to treatment, whereas citalopram did not influence anxiety levels in control SD rats, the drug robustly reduced anxiety levels in both sexes of the FSL model of depression. The anxiolytic or anxiogenic effect of different SSRI treatments on male rats has been previously reviewed thoroughly (Borsini et al., 2002) and in our study, the behaviour of control SD rats was unaffected by this particular dose of repeated SSRI treatment when rats were tested after a 24 hours wash-out period, in agreement with similar studies (Wikell et al., 1999). However, behavioural effects have also been reported under different antidepressant doses and experimental protocols (Kugelberg et al., 2002, Mombereau et al., 2010). It is worth noting that in FSL rats repeated citalopram treatment did not alter locomotor/exploratory behavioural indices but selectively affected anxiety-related behavioural responses. Interestingly, the drug-induced responses of male and female FSL rats differed in magnitude, reflecting their different baseline levels of anxiety. In light of our earlier studies (Drossopoulou et al., 2004, Dalla et al., 2005, Dalla et al., 2008, Kokras et al.,

2009a, Kokras et al., 2009b, Pitychoutis et al., 2009a), we conclude that baseline levels of anxiety in males and females of this model of depression result from sex-differentiated strategies to cope with identical behavioural challenges. Most interestingly, repeated antidepressant treatment masks such differences and produces a converging behavioural response.

This conclusion receives further support given that we only found a significant correlation between corticosterone levels and anxiety in males, although both sexes displayed reduced anxiety levels after repeated treatment with citalopram. The higher corticosterone levels found in male FSL rats most likely reflect their susceptibility to distress during handling and subsequent behavioural testing, possibly related to their impaired serotoninergic transmission (Yadid et al., 2000). Reversal of the latter by repeated antidepressant treatment attenuates stress-induced drive on the HPA axis, corticosterone and anxiety levels. On the other hand, female rats had higher corticosterone levels, as consistently reported (Dalla et al., 2010). Curiously, while female FSL also have an impaired serotoninergic system (Kokras et al., 2009a), and despite the SSRI-induced behavioural effect, treatment did not attenuate their baseline corticosterone levels. Similar sex differences in the contribution of the HPA axis to antidepressant actions have been reported (Binder et al., 2009, Horstmann et al., 2009, McEuen et al., 2009, Goel and Bale, 2010), suggesting that in female FSL (and perhaps more generally in females), additional mechanisms may influence the HPA axis, thus obscuring the correlation of corticosterone levels with behavioural outcomes following treatment.

It is known that sex differences in the regulation of corticosteroid secretion, synthesis and release of hypothalamic CRH and AVP arise during sexual organization of the brain early in life; on the other hand, the regulation of these hormones remains subject to the influence of fluctuations in gonadal secretory activity throughout life (Patchev et al., 1999). Importantly, central CRH and AVP receptors are implicated in mood and anxiety disorders and in the response to

antidepressant treatment. Here, we found that, as compared to SD rats, male and female FSL rats display lower CRHR1 receptors levels at both hypothalamic and cortical sites. Since CRHR1 has been implicated in depression in humans and mouse models (Timpl et al., 1998, Muller et al., 2003), these findings add to the view that FSL rats are a good model for understanding the neurobiological basis of depression. Interestingly, CRHR polymorphisms play an important role in the therapeutic efficacy of citalopram in anxious depression (Binder et al., 2010) and other studies have suggested interactions between CRHR1, AVPR1b and serotonin in mediating the actions of established antidepressants (Ishizuka et al., 2010, Magalhaes et al., 2010). Curiously, the results of the present study show that citalopram exerts anxiolytic actions in male and female FSL rats without altering CRHR1 expression levels. Notably, while the results of pharmacological studies in FSL rats implicate CRHR1 in the expression of depressive-like behaviour (Overstreet et al., 2004, Lavi-Avnon et al., 2005), we found here that citalopram induced changes in CRHR1 levels in SD animals even though these animals did not show behavioural responses to the drug. Offering an explanation for these apparent anomalies is difficult but the fact that FSL and SD animals show substantial differences in their baseline anxiety levels may be an important consideration. Indeed, previous studies reported that repeated SSRI treatment may affect the temporal co-localization of AVP and CRH (To et al., 1999, Moncek et al., 2003), initially affecting the CRH subsystem, and later modifying the AVP subsystem (Keck et al., 2003, Hesketh et al., 2005).

Indeed, our results show that male FSL rats, which display an anxious phenotype under baseline conditions, express higher levels of hypothalamic AVPR1b as compared to SD males. Interestingly, we also show that repeated administration of citalopram leads to a downregulation of hypothalamic AVPR1b and, in parallel, reduced corticosterone and anxiety-like behaviour in male FSL rats. In contrast, female FSL rats (non-anxious phenotype under basal conditions) exhibit lower

cortical AVPR1b mRNA levels than female SD rats. This profile was reversed by repeated citalopram treatment and the drug led to a further reduction of basal anxiety levels in female FSL rats without affecting corticosterone levels. Although the underlying mechanisms remain elusive at this time, these findings highlight the fact that the responses to citalopram are sexually differentiated in FSL animals. Given the observations that the therapeutic effects of citalopram in males are associated with a reduction in corticosterone levels, we suggest that the HPA axis plays a critical role in the regulation of anxiety in males in this sex (see Table 2). Other pathways and mechanisms appear to underlie the actions of citalopram in females; specifically, our results suggest that modulation of extra-hypothalamic (cortical) AVPR1b may be important for the anxiolytic actions of citalopram in female FSL rats (see Table 2).

In summary, our results suggest that although male and female FSL rats are a good model for research on depression, they are not suitable for accurately modelling comorbid anxious depression, as observed in humans. Interestingly though, SSRI treatment triggers distinct neurobiological mechanisms in male and female rats of this model of depression. These mechanisms result in similarly converging behavioural response in both sexes, although of different magnitude, depending on the pre-treatment baseline. It is thus suggested that similar phenotypic endpoints can arise from divergent neurobiological mechanisms, a concept previously suggested for central vasopressinergic transmission (De Vries and Panzica, 2007). It may be further suggested that the neurobiological effects of antidepressants are not strictly predetermined, but rather depend on the substrate (e.g. male or female brain, depressive and/or anxious status or not). Thus, our observations raise the question of whether a patient's clinical response to an SSRI likewise depends on sexually differentiated neurobiological mechanisms.

Acknowledgements

We thank Dieter Fischer for excellent technical assistance, Carola Hetzel for administrative help, Dr Linton for providing the CRHR1 antibody, and Drs Nuno Sousa and Alexandre Patchev for their critical comments and discussions. Citalopram was kindly gifted by Lundbeck S.A. who nevertheless did not influence the study design and interpretation of results from this work. This research was partly supported through a German-Greek Academic Exchange Programme (DAAD-IKYDA: D/04/42259), an IBRO studentship to NK, and a Max Planck Society fellowship to IS; none of these agencies had an influence over the design, outcome or interpretation of the results of this work.

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Tables

Table 1: Oestrous Cycle. Distribution of the phases of the oestrous cycle in female FSL and SD rats treated with vehicle and citalopram.

Table 2: Summary of behavioural and neurobiological changes induced by citalopram treatment on male and female FSL rats. Repeated citalopram treatment evoked an anxiolytic behavioural outcome in both male and female FSL rats but this was accompanied by hypothalamic AVPR1b and corticosterone changes in male FSL rats whereas only cortical AVPR1b changes were identified in female FSL rats.

Figures

Figure 1: Open Field: spontaneous horizontal (**a**) and vertical activity (**b**) expressed in photobeam counts, and time spent in the centre of the arena (**c**) expressed as percent of total time. Note that male but not female FSL spent less time in centre in comparison to their SD counterparts, whereas both FSL sexes responded to citalopram treatment by increasing their time spent in the centre of the arena. All data shown represent mean ± SEM values; the asterisk (*) represents a significant strain difference and the hash sign (#) represents a significant drug difference. $P<0.05$, N=6 per group.

Figure 2: Elevated Plus Maze test: number of total (**a**) and open (**b**) arm entries, and time spent in the open arms (**c**) expressed as percent of total time. Note that male but not female FSL rats spent less time in open arms in comparison to their SD counterparts but both FSL sexes responded to citalopram treatment by increasing the percentage of time spent in the open arms. All data shown represent mean ±

SEM values; the asterisk (*) represents a significant strain difference and the hash sign (#) represents a significant drug difference. P<0.05, N=6 per group.

Figure 3: Corticosterone Serum Levels

Male but not female FSL rats presented increased corticosterone serum levels and citalopram treatment successfully abolished this increase in male FSL rats (**a**). Increased corticosterone serum levels correlate with lower time in centre and open arms in males only (**b**). CORT=Corticosterone, TC=Time in Centre of the Open Field, TOA=Time in Open Arms of the Elevated Plus Mase. All data shown represent mean ± SEM values .The asterisk (*) represents a significant strain difference and the hash sign (#) represents a significant drug difference. P<0.05, N=6 per group.

Figure 4: CRHR1 and AVPR1b expression levels: Hypothalamic (**a**) and cortical (**b**) CRHR1 and hypothalamic (**c**) and cortical (**d**) AVPR1b expression levels of male and female SD and FSL rats measured by western blot analysis. Representative blots from each brain region are shown above the respective numeric data in the same order of appearance: **M**: Males, **F**: Females, **SV**: Sprague-Vehicle, **SC**: Sprague-Citalopram, **FV**: Flinder-Vehicle, **FC**: Flinder-Citalopram. Western blot numerical data are based on optical density evaluations normalized against actin and are depicted relative to control values (male vehicle-treated SD rats) as means ± SEM. An asterisk (*) represents a significant strain difference and a hash sign (#) represents a significant treatment effect. P<0.05, N=6 per group.

Figure 1

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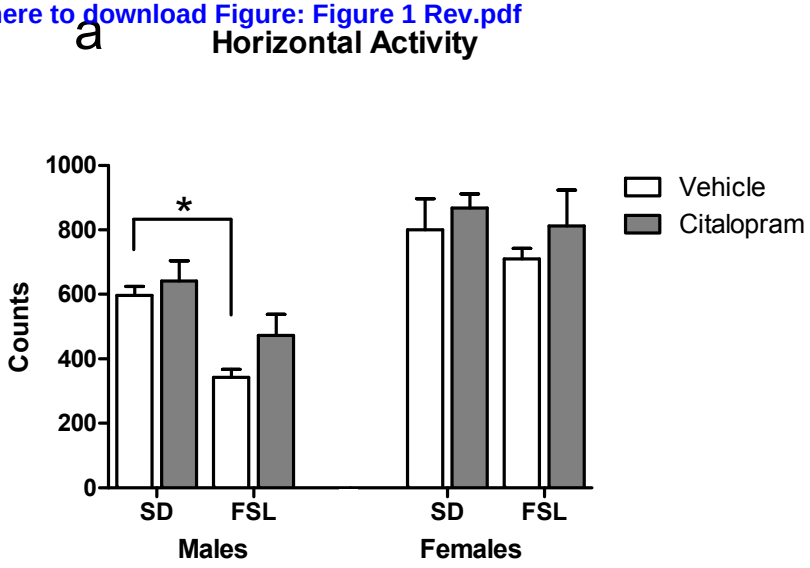

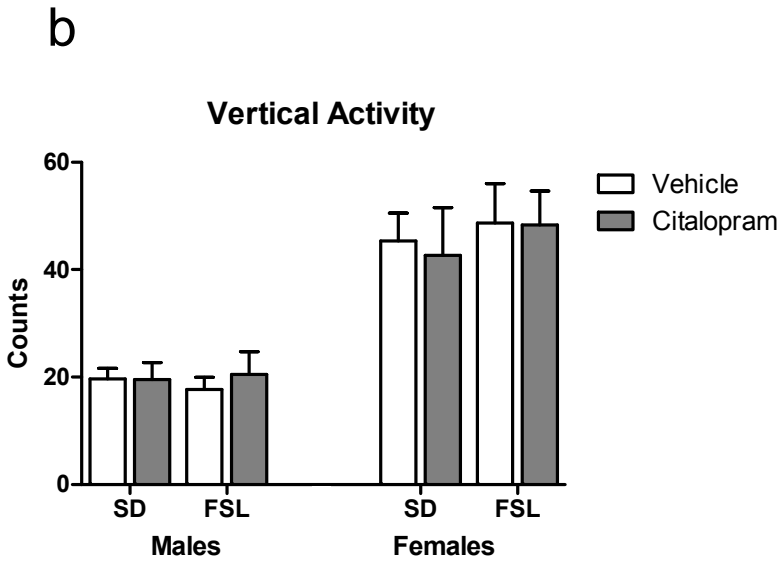

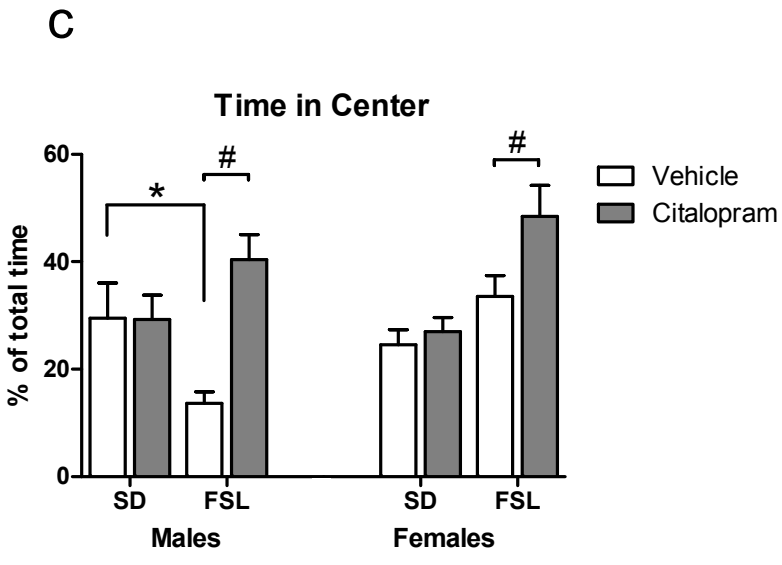

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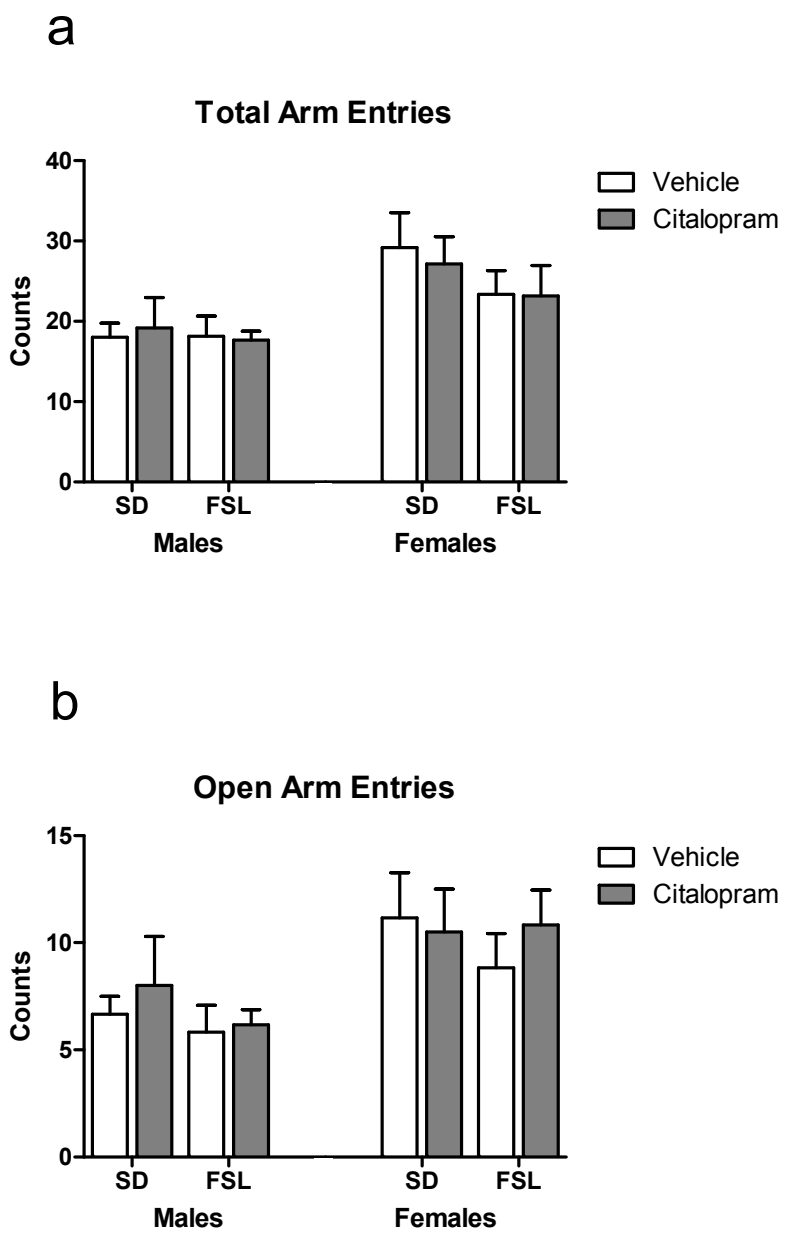

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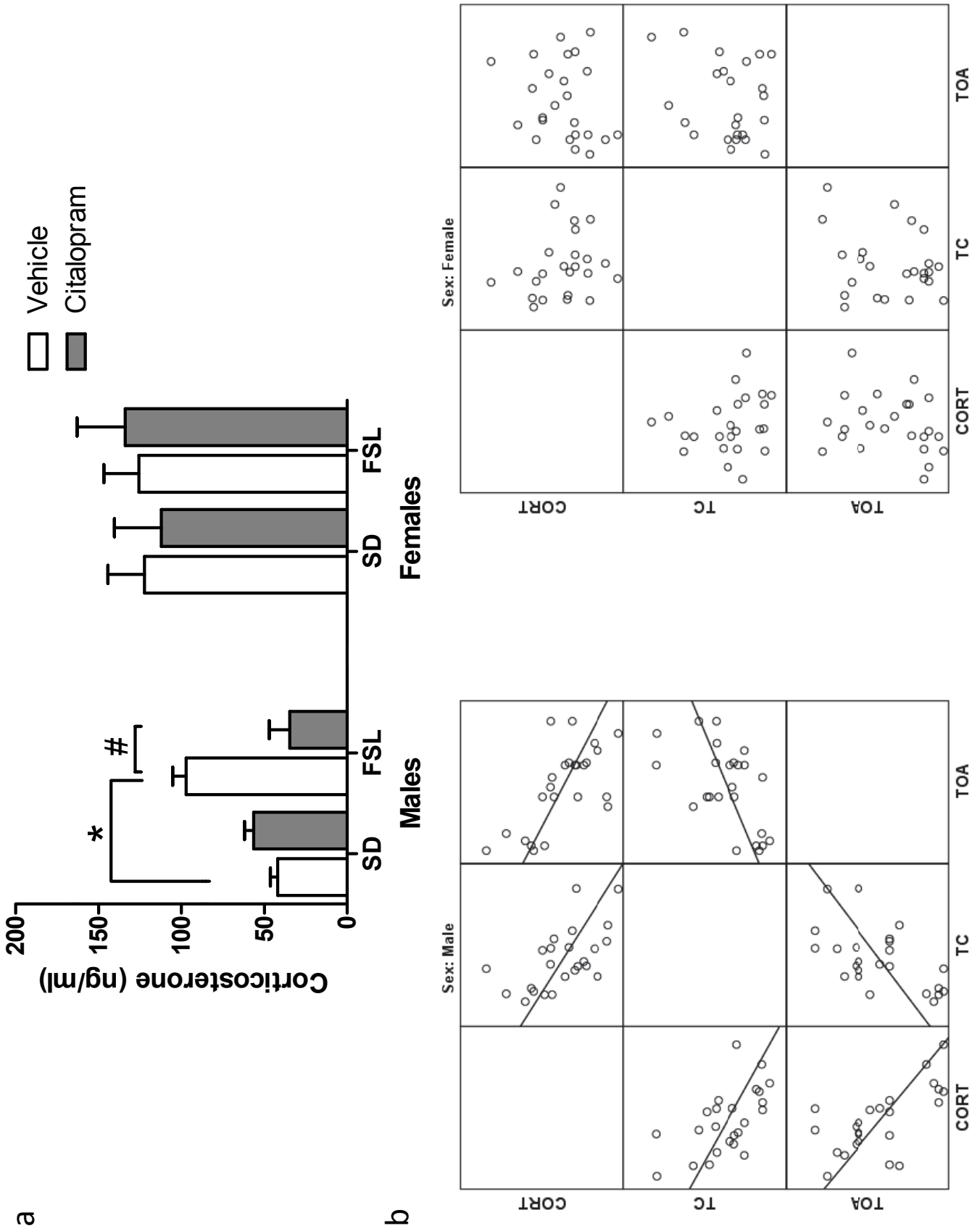

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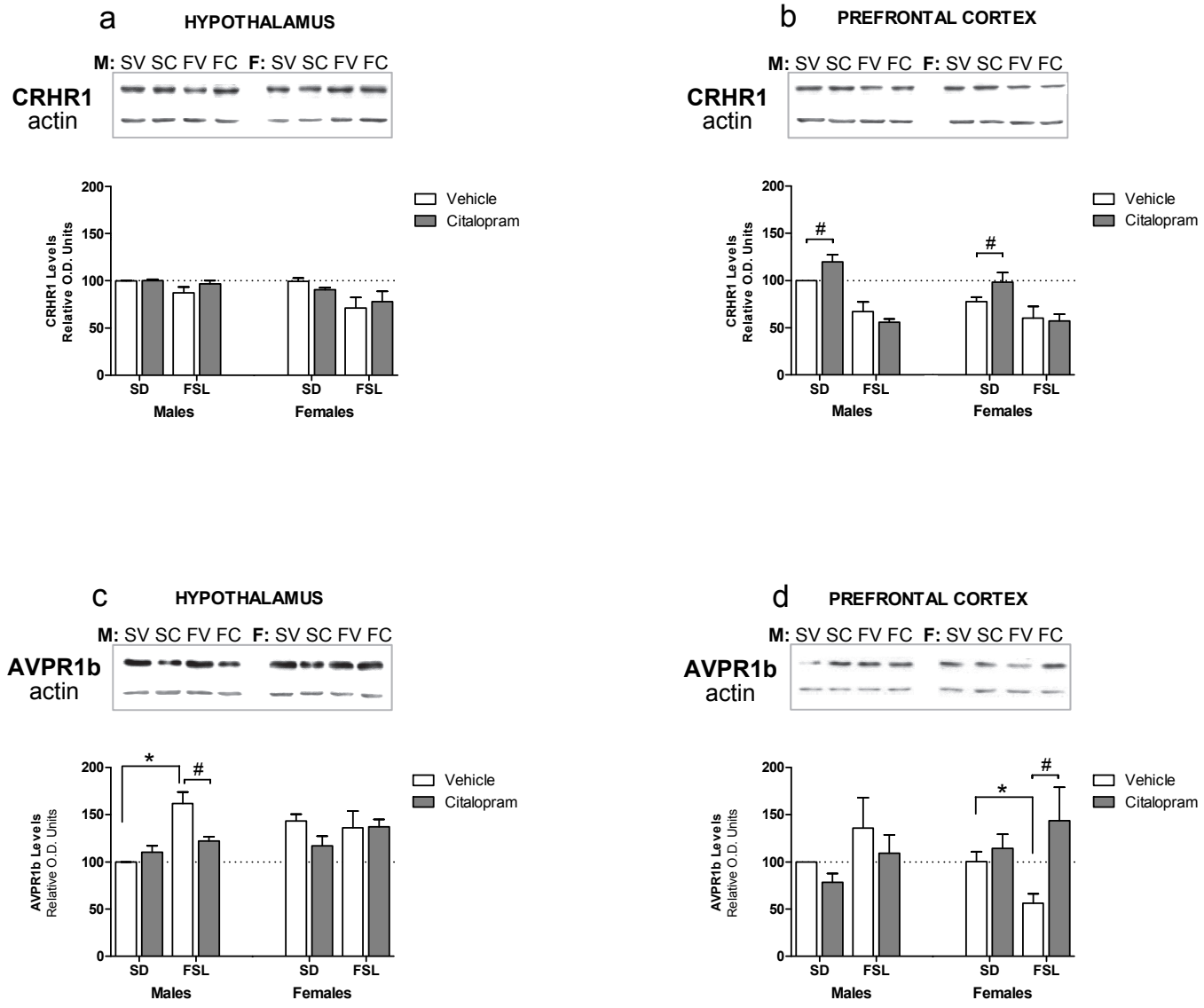

Table 1

	Vehicle		Citalopram	
	FSL	SD	FSL	SD
Proestrous	1	2	2	2
Estrous	2	1	1	1
Diestrous I	1	2	1	2
Diestrous II	2	1	2	1

Table 2

Males FSL	Anxiety	Corticosterone	HYP AVPR1b	PFC AVPR1b
Baseline	↑	↑	↑	↔
Antidepressant	↓	↓	↓	↔

Females FSL	Anxiety	Corticosterone	HYP AVPR1b	PFC AVPR1b
Baseline	↔	↔	↔	↓
Antidepressant	↓	↔	↔	↑

Article Highlights

- Male, but not female, Flinders Sensitive Line of rats present anxiety behaviour
- Repeated citalopram treatment reduces anxiety levels in both sexes
- Citalopram modulates AVPR1b expression in a sex-specific manner
- Differing mechanisms converge to produce anxiolysis in FSL rats of both sexes