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Acute stress evokes sexually dimorphic, stressor-specific patterns of neural activation across multiple limbic brain regions in adult rats

Ankit Sood, Karina Chaudhari and Vidita A. Vaidya

Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, Maharashtra, India

ABSTRACT
Stress enhances the risk for psychiatric disorders such as anxiety and depression. Stress responses vary across sex and may underlie the heightened vulnerability to psychopathology in females. Here, we examined the influence of acute immobilization stress (AIS) and a two-day short-term forced swim stress (FS) on neural activation in multiple cortical and subcortical brain regions, implicated as targets of stress and in the regulation of neuroendocrine stress responses, in male and female rats using Fos as a neural activity marker. AIS evoked a sex-dependent pattern of neural activation within the cingulate and infralimbic subdivisions of the medial prefrontal cortex (mPFC), lateral septum (LS), habenula, and hippocampal subfields. The degree of neural activation in the mPFC, LS, and habenula was higher in males. Female rats exhibited reduced Fos positive cell numbers in the dentate gyrus hippocampal subfield, an effect not observed in males. We addressed whether the sexually dimorphic neural activation pattern noted following AIS was also observed with the short-term stress of FS. In the paraventricular nucleus of the hypothalamus and the amygdala, FS similar to AIS resulted in robust increases in neural activation in both sexes. The pattern of neural activation evoked by FS was distinct across sexes, with a heightened neural activation noted in the prelimbic mPFC subdivision and hippocampal subfields in females and differed from the pattern noted with AIS. This indicates that the sex differences in neural activation patterns observed within stress-responsive brain regions are dependent on the nature of stressor experience.

1. Introduction
Stress contributes to the precipitation and exacerbation of psychiatric disorders such as anxiety and depression (Chrousos, 2009; Cohen, Janicki-Deverts, & Miller, 2007). Clinical studies indicate that disorders such as depression, anxiety, and post-traumatic stress disorder are almost twice as common in women (Kessler, 2003; Kessler et al., 2005). While the underlying mechanisms for these differences in vulnerability to psychopathology remain unclear, it raises the possibility that stress is perceived and processed differently in the male and female brain (Bangasser & Valentino, 2014). Indeed, preclinical studies demonstrate that the sex of an individual, prior life history, as well as the severity, frequency, and duration of stressor experience can influence the nature of stress response (Anisman & Matheson, 2005). Stress responses involve coordination between multiple central and peripheral systems, primary amongst which is the activation of the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis, which involves stimulation of the paraventricular nucleus (PVN) of the hypothalamus, and the release of adrenal stress hormones that exert their hormonal actions via corticosteroid receptors in both peripheral and central target regions (Radley, Gosselink, & Sawchenko, 2009). HPA axis function, both under baseline conditions and in response to stress, is known to exhibit differences between males and females, with studies in female rats indicating higher baseline and acute stress-evoked circulating corticosterone levels (Babb, Masini, Day, & Campeau, 2013a,b).

Although the primary initiation of the stress response involves activation of the PVN, multiple limbic brain regions including higher order cortical brain regions such as the medial prefrontal cortex (mPFC) and hippocampus, as well as subcortical neurocircuits such as the lateral septum (LS), habenula, and amygdala are also activated in response to acute stress (Cullinan, Helmreich, & Watson, 1996; Cullinan, Herman, Battaglia, Akil, & Watson, 1995). These brain regions have been implicated in the processing of stress-coping strategies, aversive, avoidance, and depressive behaviors, fear responses, as well as in the top-down regulation of the HPA axis contributing to the termination or prolonging of neuroendocrine stress responses (Mcklveen, Myers, & Herman, 2015; Ulrich-Lai & Herman, 2009; Vermetten & Bremner, 2002). Prior rodent studies suggest sex differences in coping strategy, with a bias towards passive behavioral stress responses noted in females (Drossopoulou et al., 2004). Further, females have been reported to exhibit enhanced despair behavior in responses to acute and short-term stressors.
(Dalla, Antoniou, et al., 2008; Drossopoulou et al., 2004). Interestingly, while helplessness behavior appears to predominantly be manifested in males when exposed to uncontrollable stress, effects of shock exposure on associative learning, and spine architecture are observed in females (Dalla, Edgecomb, Whetstone, & Shors, 2008; Shors, Chua, & Falduto, 2001; Wood & Shors, 1998). In addition, acute restraint stress is reported to impair spatial memory in males and result in an opposing enhancement in females (Conrad et al., 2004).

Given the important role brain regions such as the mPFC, hippocampus, LS, habenula, and amygdala play in both the perception and processing of stress, and the modulatory control they exert on the PVN and HPA axis (López, Akił, & Watson, 1999; Ulrich-Lai & Herman, 2009), it is important to understand whether these brain regions are differentially activated by acute or short-term stress in the male and female brain. While we have restricted ourselves to examining forebrain structures implicated in stress responses, it is important to note that brainstem structures such as the raphe nuclei, ventral tegmental area, locus coeruleus, and periaqueductal gray also contribute to stress responses (Chrousos, 2009).

To investigate potential sex differences in neural activation within specific stress-associated neurocircuits, we subjected male and female rats to the well-known psychogenic stress paradigm of acute immobilization stress (AIS) or to the short-term stress of forced swim (FS). We used Fos immunopositivity as a marker for neural activation (Kovács, 2008) and examined the influence of AIS and FS on Fos positive cell numbers in the mPFC, hippocampus, LS, LHb, PVN and basolateral (BLA), central (CeA), and basomedial (BMA) amygdaloid nuclei in the male and female rat brain. Our results indicate that while AIS evokes sexually dimorphic neural activation patterns in both cortical and subcortical brain regions, FS results in sex differences in neural activation only within cortical circuits. These findings indicate that neural activation of brain regions implicated in stress responses differs in the male and female brain and also exhibits stressor-specificity.

2. Material and methods

2.1. Animals

Adult age-matched male and female Sprague-Dawley rats were used for all experiments. Animals were either bred at the TIFR animal facility or purchased from Reliance Healthcare and housed in the TIFR animal facility. Animals were group housed (3–4 animals per cage) and maintained on a 12:12 h light: dark cycle (lights on at 7:00 a.m.) with ad libitum access to food and water. Animals were assigned at random to control, acute immobilization stress (AIS), or forced swim stress (FS) groups. All experimental procedures followed the National Guidelines of the Committee for Supervision and Care of Experimental Animals (CPCSEA) and were approved by the TIFR Institutional Animal Ethics committee.

2.2. Stress paradigms

For the AIS experiments, animals were placed in plastic restrainer cones (Harvard Apparatus) that were taped at the base to minimize movement and then kept in a cage for a duration of two hours. For the FS, on day 1 animals were placed in clear cylindrical tanks (60 × 30 cm) with 23 °C water filled up to a height of 40 cm and allowed to swim for 15 min. Later, animals were taken out, dried, and returned to their home cages. On day 2, animals were placed in the tank for a duration of 5 min under identical conditions. All control animals were maintained in their home cages and left undisturbed.

2.3. Immunohistochemistry

Animals were sacrificed two hours after commencement of the AIS procedure or two hours after forced swim exposure on day 2 of the FS. Controls were left undisturbed and sacrificed on the same day as the stress groups. Animals were transcardially perfused with 0.9% ice-cold saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Brains were removed and stored in 4% PFA until sectioning. Coronal sections (40 μm) were cut using a vibratome (Leica, Germany) and stored in 0.1 M PB. For Fos immunohistochemistry, sections were blocked in 10% horse serum in 0.3%PbTx (0.3% Triton X 100 in 0.1 M PB) for 2 h followed by overnight incubation with the primary antibody (Rabbit anti Fos; 1:1000; CST, USA) in 0.1 M PB at room temperature. Following washes, sections were incubated with secondary antibody (biotinylated Donkey anti Rabbit; 1:250; Millipore, Billerica, MA) at room temperature for 3 h. Following incubation with avidin-biotin complex solution (Vectastain Elite ABC HRP kit, Vector labs, Burlingame, CA) and signal visualization with Diaminobenzidine (DAB, Sigma, St. Louis, MO), sections were examined under a bright field microscope (Zeiss Axioskop 2 plus, Carl Zeiss, Germany).

2.4. Cell counting

Cell counting analysis to assess the number of Fos immunopositive cells was performed by an observer blind to the experimental treatment conditions. Fos immunopositive cells were counted using a 20X objective under a bright field microscope (Zeiss Axioskop 2). Cells were considered to be Fos immunopositive only when they exhibited strong labeling. The regions of the brains analyzed were: mPFC (six sections per animal, every fourth section), LS (five sections per animal, every fourth section), amygdala (BLA, CeA, and BMA) and PVN (4–5 sections per animal, every sixth section), lateral habenula (LHb) (3–4 sections per animal, every sixth section) and the dorsal hippocampus (six sections per animal, every sixth section). The cingulate (Cg), prelimbic (PL), and infralimbic (IL) subdivisions of the mPFC were identified with the help of the rat brain atlas (Paxinos & Watson, 1998). The total number of cells counted per region per animal was divided by the number of sections to obtain a final count of the average number of cells per section for that brain region. A mean number of cells per section for specific brain regions was determined across all animals analyzed and results were expressed as mean ± SEM.
2.5. Statistical analysis

Data were analyzed using the software Prism 5.0 (Graphpad Inc, La Jolla, CA). Data were tested for normality using the Kolmogorov-Smirnov test (KS test). Two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc multiple comparisons across groups was used to analyze the results. Significance was set at \( p < .05 \). Pair-wise Pearson correlation coefficients (\( r \)) were calculated for Fos positive cell numbers to assess the extent of correlation in neural activation patterns across brain regions following AIS or FS in males and females.

3. Results

3.1. Acute immobilization stress evokes sexually dimorphic patterns of Fos expression in the prefrontal cortex and hippocampus

The mPFC and the hippocampus are brain regions that are targets for stress hormones and also play a key role in modulating stress-coping behavior, avoidance responses, and feedback regulation of neuroendocrine stress responses by exerting control on the HPA axis (Ulrich-Lai & Herman, 2009). Here, we examined whether the AIS-evoked pattern of neural activation in the mPFC subdivisions (Cg, PL and IL) and hippocampal subfields (DG, CA1, and CA3), as measured through assessing the expression of the neural activity marker Fos, differed between the males and females (Figure 1(a,b)).

Analysis of Fos immunopositive cell numbers within the mPFC subdivisions of male and female rats subjected to AIS, revealed a sexually dimorphic pattern of Fos expression in the Cg (Figure 1(d)) and IL (Figure 1(c,f)), but not the PL (Figure 1(e)). Two-way ANOVA analysis for Fos within the Cg indicated a significant AIS×Sex interaction (Figure 1(d)) (\( F(1,34) = 10.43, p < .002 \), as well as a significant main effect of AIS (\( F(1,34) = 36.53, p < .001 \)) but not sex (Figure 1(d)). Bonferroni post-hoc comparisons indicated that males exhibited a significant increase in Fos cell numbers within the Cg following AIS (Figure 1(d)). This pattern was distinct in females, which did not exhibit a similar extent of an AIS-evoked increase in Fos cell numbers in the Cg. In contrast, for Fos-positive cell numbers within the PL subdivision of the mPFC (Figure 1(e)), two-way ANOVA analysis indicated only a main effect of stress (\( F(1,35) = 50.47, p < .001 \)), with no significant sex effect, or AIS×Sex interaction. Bonferroni post-hoc comparisons indicated that the extent of AIS-evoked increase in the number of Fos positive cells within the PL did not differ between males and females (Figure 1(e)). Further, two-way ANOVA analysis for Fos cell counts in the IL (Figure 1(c,f)) also indicated a significant interaction of AIS×Sex (\( F(1,34) = 15.65, p < .001 \)), as well as significant main effect of both AIS (\( F(1,34) = 82.31, p < .001 \)) and sex (\( F(1,34) = 20.05, p < .001 \)). Bonferroni post-hoc comparisons indicated that while the number of Fos positive cells was increased in the IL of both males and females post AIS as compared to their sex-matched controls, the extent of AIS-evoked Fos expression was significantly higher in males versus females (Figure 1(c,f)).

We next examined the influence of AIS on Fos-positive cell numbers in the DG, CA1, and CA3 hippocampal subfields of both male and female rats (Figure 1(g–j)). Two-way ANOVA analysis for Fos positive cell numbers in the DG revealed a significant AIS×Sex interaction (\( F(1,37) = 13.03, p < .001 \)) and a main effect of stress (\( F(1,37) = 17.57; p < .001 \)) but not sex (Figure 1(g,h)). Bonferroni post-hoc comparisons indicated that while male rats exposed to AIS did not show any change in the number of Fos positive cells as compared to non-stressed male controls, females subjected to AIS exhibited a significant decline in the number of Fos positive cells as compared to both non-stressed sex-matched controls and AIS administered males (Figure 1(h)). Within the CA1 subfield (Figure 1(i)), two-way ANOVA analysis revealed a significant AIS×Sex interaction (\( F(1,37) = 10.37, p = .002 \)) and a main effect of AIS (\( F(1,37) = 8.244; p = .006 \)). Two-way ANOVA analysis for Fos cell counts in the CA3 subfield (Figure 1(j)) also indicated a significant AIS×Sex interaction (\( F(1,37) = 6.746; p = .013 \)) along with a main effect of AIS (\( F(1,37) = 7.790; p = .008 \)). Bonferroni post-hoc comparisons indicated that unlike the DG, AIS led to a significant increase in the number of Fos positive cells in the males in both CA1 (Figure 1(i)) and CA3 (Figure 1(j)) subfields as compared to their sex matched controls. In contrast, female rats failed to show any change in Fos-positive cell numbers in both the CA1 and CA3 following AIS (Figure 1(i,j)). Taken together, these results indicate that AIS evokes sex-dependent distinct patterns of neural activation within specific mPFC subdivisions (CG and IL) and in all the hippocampal subfields.

3.2. Acute immobilization stress evokes sexually dimorphic patterns of Fos expression in specific stress-responsive, subcortical brain regions

We next sought to address whether the sexually dimorphic pattern of AIS evoked neural activation, noted via Fos counting analysis, in cortical brain regions of the mPFC, and hippocampus, also extended to subcortical stress-responsive circuits. We assessed the influence of AIS on the numbers of Fos-positive cells within the LS and the LHb (Figure 2(a–f)), the PVN of the hypothalamus and the BLA, CeA, and BMA amygdaloid nuclei (Figure 3(a–h)).

AIS resulted in a sexually dimorphic pattern of Fos expression in both the LS and LHb, with a significantly higher magnitude of Fos induction noted in the AIS males. In the LS (Figure 2(c,d)), two-way ANOVA analysis revealed a significant AIS×Sex interaction (\( F(1,38) = 5.574; p = .023 \)), as well as a significant main effect of both sex (\( F(1,38) = 6.308; p = .016 \)) and AIS (\( F(1,38) = 41.55; p < .001 \)). Bonferroni post-hoc comparisons indicated that while both males and females exhibited significant increases in the number of Fos positive cells in the LS as compared to their own sex-matched controls following AIS, the extent of Fos induction in the males was significantly greater than that observed in the LS in females (Figure 2(c,d)). Two-way ANOVA analysis for Fos positive cell numbers in the LHb (Figure 2(e,f)) indicated a significant AIS×Sex interaction (\( F(1,35) = 6.682; p = .014 \), with a main effect observed for AIS (\( F(1,35) = 36.83; p < .001 \)). Bonferroni post-hoc comparisons revealed that while both males and females showed enhanced Fos cell numbers in the LHb following AIS,
they differed in the degree of induction with the females showing a lesser extent of neural activation (Figure 2(e,f)).

In contrast to the sexually dimorphic neural activation pattern noted in the LS and LHb following AIS, the numbers of Fos positive cells induced by AIS in the PVN and specific amygdaloid nuclei did not differ across the sexes. Fos cell counting analysis within the BLA, CeA, and BMA (Figure 3(c–f)), revealed that the extent of Fos induction following AIS did not differ between sexes. Two-way ANOVA analysis of Fos in the BLA revealed main effects of AIS (Figure 3(c,d)) ($F_{(1,25)} = 43.92, p < .001$) and no AIS x Sex interaction. Within the CeA, we noted a main effect of both AIS (Figure 3(e)) ($F_{(1,25)} = 32.14, p < .001$) and sex ($F_{(1,25)} = 20.25, p < .001$) but no significant AIS x Sex interaction. Two-way analysis of Fos cell numbers within the BMA revealed a main effect of stress (Figure 3(f)) ($F_{(1,25)} = 17.27, p < .001$) and no significant AIS x Sex interaction. Bonferroni post-hoc comparisons indicated that AIS induced significant increases in Fos-positive cell numbers in the BLA, CeA, and BMA of both sexes (Figure 2(i,j)). Similarly, Two-way ANOVA analysis of Fos-positive cell numbers in the PVN (Figure 3(g,h)) indicated only a main effect of AIS ($F_{(1,25)} = 26.60; p < .001$) and no AIS x Sex interaction. Bonferroni post-hoc comparisons indicated that AIS evoked a similar extent of increase in the number of Fos positive cells in the PVN of both male and female rats as compared to their sex-matched controls (Figure 3(g,h)).
Collectively, these findings reveal that AIS evokes sexually distinct patterns of neural activation within specific, stress-responsive subcortical brain regions namely the LS and LHb, but not in the PVN and amygdala.

### 3.3. Acute immobilization stress evokes sexually dimorphic patterns of neural activation in cortical and subcortical brain regions

AIS evoked sexually dimorphic patterns of neural activation within multiple stress-responsive cortical (mPFC and hippocampus) and subcortical (LS and LHb) brain regions (Figure 4). Fos counting analysis indicated that both the pattern and extent of neural activation varies between the male and female brain, suggestive of the fact that the stressor stimulus may be perceived and processed differentially based on sex. The results observed in Figures 1–3 are summarized via a schematic (Figure 4) that indicates the pattern of neural activation following AIS in both the male and female brain.

Within the mPFC, AIS evoked increased neural activation in all subdivisions, in contrast in the female mPFC AIS enhanced neural activation only in the PL and IL. Further, the magnitude of AIS-induced neural activation was higher in the Cg and IL, but not the PL, of male rats (Figure 4). The pattern of neural activation in the hippocampus following AIS differed starkly between the male and female brain, with a decline in DG neural activity noted only in the female brain, and an increase in neural activity in the CA1 and CA3 noted only in the male brain (Figure 4). Within the subcortical brain regions the pattern of neural activation did not vary across males and females in the primary stress-responsive hypothalamic nucleus, the PVN, and also in the amygdala (Figure 4). In contrast, within both the LS and LHb we noted a greater magnitude of neural activation in the male versus female brain following AIS.

### 3.4. Forced swim stress evokes sexually dimorphic patterns of Fos expression in the medial prefrontal cortex

We next sought to examine whether the sexually dimorphic pattern of neural activation in response to AIS was stressor-dependent. We used FS to examine whether a short duration exposure to swim stress across two days resulted in different patterns of neural activation in male versus female rats. We subjected male and female rats to FS and examined the numbers of Fos positive cells in the same brain regions that were analyzed following AIS, to gain a comparative understanding of the pattern of neural activation (Figure 5(a,b)).

Counting analysis of Fos positive cell numbers in the Cg, PL, and IL subdivisions of the mPFC of male and female rats following FS, indicated a dimorphic pattern of Fos expression only within the PL (Figure 5(e)), but not the Cg (Figure 5(d)) and IL (Figure 5(c,f)). Two-Way ANOVA analysis for Fos cell numbers in the Cg (Figure 5(d)) revealed significant main effects of both FS ($F_{(1,33)} = 38.69; p < .001$) and sex ($F_{(1,33)} = 5.335; p = .027$) but no FS × Sex interaction. Bonferroni post-hoc comparisons revealed that while both males and females had increased number of Fos positive cells post FS exposure in the Cg, there was no significant
difference between the extent of neural activation in males and females (Figure 5(d)). In the PL subdivision of the mPFC (Figure 5(e)), two-way ANOVA analysis indicated a significant FS × Sex interaction ($F_{(1,33)} = 7.951; p = .008$) along with significant main effects for both FS ($F_{(1,33)} = 52.50; p < .001$) and sex ($F_{(1,33)} = 13.59; p < .001$). Bonferroni post-hoc comparisons revealed that FS led to an increase in the number of Fos positive cells in the PL of both males and females, with the magnitude of increase in Fos-positive cells in females being significantly greater as compared to FS males (Figure 5(e)).

Two-way ANOVA analysis for Fos numbers in the IL (Figure 5(c,f)) indicated a significant main effect of FS ($F_{(1,33)} = 67.62; p < .001$), but no FS × Sex interaction. Bonferroni post-hoc comparisons indicated that both males and females had increased number of Fos positive cells within the IL after FS exposure as compared to respective sex-matched controls (Figure 5(f)). Our results indicate that FS results in a clearly differing pattern of neural activation in the prelimbic subdivision of the male and female mPFC.

Within the hippocampus, two-way ANOVA analysis for Fos positive cell numbers did not indicate FS × Sex interaction in the DG (Figure 5(g,h)), CA1 (Figure 5(i)), or CA3 (Figure 5(j)) hippocampal subfields. In the DG subfield, we noted significant main effects of both FS ($F_{(1,34)} = 5.2, p = .02$) and sex ($F_{(1,34)} = 19.23, p < .001$) (Figure 5(g,h)). Bonferroni post-hoc comparisons indicated that while males did not show any significant change in the number of Fos positive cells following FS, females exhibited a robust increase in Fos cell number.
Within the CA1 hippocampal subfield (Figure 5(i)), we observed a main effect of FS ($F(1,34) = 18.16; p < .001$) and sex ($F(1,34) = 9.96, p = .003$). Bonferroni post-hoc comparisons revealed that females and not males displayed a significant induction of Fos positive cell numbers following FS as compared to their sex-matched controls (Figure 5(i)).

Two-way ANOVA analysis for Fos positive cell numbers in the CA3 (Figure 5(j)) revealed a main effect of FS ($F(1,34) = 36.15; p < .001$) but not for sex. Bonferroni post-hoc comparisons indicated that the number of Fos positive cells was increased in both males and females following FS as compared to their sex-matched, non-stress controls (Figure 5(j)). The magnitude of the FS-evoked increase in Fos positive cell numbers in the CA3 did not differ between males and females.

### 3.5. Forced swim stress evoked pattern of neural activation in stress-responsive, subcortical brain regions does not vary across sexes

We next assessed whether the neural activation pattern following FS noted in the LS, LHb (Figure 6(a–f)), PVN and BLA, CeA, and BMA (Figure 7(a–h)) differed across sexes. Two-way ANOVA analysis for Fos positive cell numbers did not indicate any FS × Sex interaction for any of the subcortical brain regions.
regions analyzed. We observed a significant main effect for FS in the LS (Figure 6(c,d)) ($F_{(1,39)} = 75.68; p < .001$) and the LHb (Figure 6(e,f)) ($F_{(1,30)} = 83.14; p < .001$). Bonferroni post-hoc comparisons revealed that both males and females subjected to FS showed increased number of Fos positive cells in the LS (Figure 6(d)) and LHb (Figure 6(f)) as compared to their sex-matched non-stressed controls and the magnitude of this increase was similar in males and females. Two-way analysis of Fos numbers within the BLA (Figure 7(c,d)) revealed a main effect of FS ($F_{(1,27)} = 38.88, p < .001$) but no significant $FS \times Sex$ interaction. Within the CeA (Figure 7(e)) and BMA (Figure 7(f)), two-way analysis revealed main effects of both FS (CeA: $F_{(1,27)} = 19.57, p < .001$; BMA: $F_{(1,27)} = 60.12, p < .001$) and sex (CeA: $F_{(1,27)} = 15.52, p < .001$; BMA: $F_{(1,27)} = 10.43, p < .001$) and no significant $FS \times Sex$ interaction. Within the PVN (Figure 7(g,h)), two-way ANOVA analysis indicated significant main effects of both FS ($F_{(1,28)} = 44.42; p < .001$) and sex ($F_{(1,28)} = 4.899; p = .0352$). Bonferroni post-hoc comparisons indicated that both male and female rats subjected to FS exhibited a significant increase in the number of Fos positive cells in the amygdala (Figure 7(c-f)) and the PVN (Figure 7(g,h)) as compared to sex-matched non-stressed controls. The extent of FS-evoked increase in the number of Fos positive cells in the PVN and amygdala did not differ between males and females. Collectively, these observations indicate FS does not result in any differences in either the pattern of neural activation or the magnitude of increases in Fos cell numbers within the LS, LHb, PVN, and amygdala.
3.6 Forced swim stress evokes sexually dimorphic patterns of neural activation in cortical, but not subcortical, brain regions

Forced swim stress resulted in a sex-dependent differential pattern of neural activation within specific cortical brain regions, with no sex difference was noted in the pattern or degree of neural activation in the multiple subcortical brain regions examined. The results observed in Figures 5–7 have been summarized through a schematic (Figure 8) that reveals the pattern of neural activation following FS in the male and female brain. FS resulted in neural activation in all the mPFC subdivisions with a similar extent of regulation noted for the Cg and IL in both males and females (Figure 8). Within the PL subdivision of the mPFC, FS resulted in a higher magnitude of neural activation in female rats. While two-way ANOVA analysis did not indicate any FS × Sex interactions for any of the hippocampal subfields, we did note that FS resulted in robust increases in neural activation in the DG and CA1 hippocampal subfields of female rats, a pattern that was not observed in males (Figure 8). In the CA3 hippocampal subfield, FS resulted in a similar increase of neural activation in both males and females subjected to FS. Within the subcortical brain regions, the pattern of FS-evoked neural activation did not vary across males and females in the LS, LHb, PVN, and also in the BLA, CeA, and BMA (Figure 8). FS resulted in an increase in the Fos positive cell numbers in all the four regions analyzed, with the extent of increase being similar between males and females across all the four regions.

Discussion

Our results provide novel evidence that AIS and FS evoke sex-dependent, distinct Fos expression changes in multiple brain regions, suggestive of differing patterns of neural activation in stress-processing circuitry in the male and female brain. This sexual dimorphism in stress-evoked Fos expression was reflected both in the extent and pattern of Fos induction and further indicated that these differences following stress vary based on the nature of stressor.

Previous reports indicate that diverse acute stressors increase Fos expression within all subdivisions of the mPFC (Cullinan et al., 1995). Our results also reveal that both AIS and FS evoke enhanced Fos positive cell numbers in the Cg, PL, and IL subdivisions of the mPFC of both males and females, albeit to differing extents and in a stressor-specific fashion. While AIS resulted in a greater extent of activation of Fos expression in both the Cg and IL in the male brain, FS resulted in a higher degree of Fos expression in the PL in the female brain. These findings highlight not only the differing functional activation patterns in response to acute stress in the male and female brain, but also serve to underscore the importance of the nature of stressor. Recent findings indicate that passive and active coping strategies exhibited during the two days of FS exhibit variation across sexes in a strain-dependent manner (Colom-Lapetina et al., 2017). Prior evidence implicates mPFC hyperactivity in contributing to both the adaptive and maladaptive responses to stress (Covington et al., 2010; Wang, Perova, Arentiikel, & Li, 2014). Studies based on optogenetic or high-frequency electrical mPFC stimulation provide evidence for both increased and attenuated depressive behavior (Covington et al., 2010; Hamani et al., 2010; Warden et al., 2012; Vizhae et al., 2011). This suggests that activation of distinct mPFC neuronal subpopulations that project to diverse cortical and subcortical stress-response circuitry could exert diametrically opposing effects on stress responses (Radley, Arias, & Sawchenko, 2006). Studies that
have targeted individual pathways support a role for mPFC input to the LHb or BLA in enhancing despair-like behavior (Amat et al., 2001; Li et al., 2011; Martinez et al., 2013; Moscarello & LeDoux, 2013; Warden et al., 2012), whereas mPFC projections to the dorsal raphe nucleus (Warden et al., 2012) enhance resilient responses and activation of the mPFC-accumbens pathway contributes to recovery from anhedonia and social avoidance (Vialou et al., 2014). However, most of these studies have been carried out in males thus raising the question as to whether stress-processing within the mPFC of the female brain adopts a similar strategy or recruits distinct mPFC subpopulations biasing responses in a sex-dependent fashion. Estrogen is known to exert strong effects on mPFC neuronal morphology (Hao et al., 2007) and has also been shown to interact with stress to influence dendritic remodeling within BLA-projecting mPFC neurons suggesting that stress-processing cortical circuitry may be differentially regulated in males and females (Shansky et al., 2010). Given reports of differences in anhedonic, avoidance, and despair-like behavior that arise in males and females following acute stress (Dalla, Antoniou, et al., 2008; Dalla, Edgecomb, et al., 2008), it is important to address whether stress processing differs in the recruitment of mPFC circuitry in males and females. While our results do not allow us to parcellate out effects of AIS or FS on specific mPFC subpopulations that target distinct downstream brain regions,
they highlight clear differences in the extent and pattern of Fos activation within the mPFC subdivisions and raise the intriguing possibility that such differences may contribute to biases in stress-processing based on sex.

We also observed a completely differing effect of AIS and FS on Fos expression within the hippocampal subfields. Our findings indicated that AIS results in a significant decline in Fos cell numbers within the DG of only females, whereas AIS enhances Fos expression in the CA1 and CA3 of only males. Interestingly, while we did not observe any interaction effects of FS and sex in the hippocampal subfields, we did observe a pattern suggestive of enhanced Fos expression in the female DG and CA1 in response to FS. A previous report indicates lower Fos mRNA levels in proestrous and estrous females subjected to acute restraint as compared to males (Figueiredo, Dolgas, & Herman, 2002). This has been speculated to contribute to differential extent of inhibition of the HPA axis, as the hippocampus exerts a top-down multi-synaptic inhibitory feedback on the PVN (Radley & Sawchenko, 2011). Females are known to exhibit higher stress-evoked corticosterone and differences in extent of activation of the hippocampus could contribute to an attenuated feedback inhibition of the HPA axis in females (Babb et al., 2013). Indeed, studies suggest that the extent of hippocampal Fos expression is negatively correlated with circulating corticosterone levels (Goel, Plyler, Daniels, & Bale, 2011), this would then raise the possibility that the AIS evoked decrease in Fos restricted to the female DG may underlie the differences in the extent of feedback regulation of the HPA axis in females following acute stress. Previous reports based on voltage sensitive dye imaging studies highlight relative reductions in DG activity as compared to CA1 activity as a signature associated with enhanced despair-like behavior in female rats subjected to chronic mild stress, with a reversal in this pattern linked to

Figure 8. Sexual dimorphism in the pattern of neural activation evoked by forced swim stress across multiple brain regions. Shown is a schematic summarizing the influence of forced swim stress (FS) on the number of Fos positive cells within the cortical and subcortical brain regions represented in rostrocaudal order, namely the medial prefrontal cortex (mPFC), lateral septum (LS), paraventricular nucleus of the hypothalamus (PVN), amygdala (AMY) which includes the basolateral, central and basomedial nuclei, the lateral habenula (LHb), and the hippocampus (HPC) in the male and female brain. Within the mPFC, the schematic illustrates dimorphic effects on Fos cell numbers across the subdivisions, namely the cingulate (Cg), prelimbic (PL), and infralimbic (IL) cortices and in the hippocampal subfields of the dentate gyrus (DG), CA1, and CA3. We observed a sexual dimorphism in the FS-evoked Fos expression pattern only in the PL subdivision of the mPFC, where females had significantly more Fos positive cells than males following FS. Also of interest is the hippocampus, where we observed that while males failed to show any change in the number of Fos positive cells in the DG and CA1 subfields following FS, females exhibited an increase in Fos positive cell numbers across all three hippocampal subfields. Included is a color key indicating the nature of change in Fos positive cell numbers in the male and female brain.
antidepressant-like behavioral responses (Airan, Meltzer, & Deisseroth, 2007). We also find that at least in the context of the AIS model of stress, females exhibit a significant decrease in DG activity suggesting that this could contribute to a decreased ratio of DG-CA1 activity, which may impact hippocampal network activity and potentially contribute to increased risk for stress-evoked depressive behaviors observed in females (Airan et al., 2007). However, it is important to note that a short-term stress of FS evokes a completely differing pattern of Fos expression in the hippocampus as compared to AIS, and this highlights the need to understand both the stressor-specificity and the influence of number of stressor exposures prior to reaching any general conclusion of sex-dependent patterns of Fos activation in the female hippocampus. Previous findings suggest that while the stress of forced swimming affects synaptic excitability in both male and female rats, intrinsic excitability, and frequency-dependent inhibition are only altered in males, suggesting that at the level of electrophysiological changes in the DG males appear more sensitive to stress (Zitman & Richter-Levin, 2013).

Examination of the pattern of neural activity evoked by AIS and FS in subcortical stress-response neurocircuitry, namely the LS, LHb, PVN, and amygdala, indicated only a significant AIS × Sex interaction within the LS and LHb. We observed that both AIS and FS led to a robust increase in the number of Fos positive cells in the LS and LHb of males as well as females. Interestingly, the extent of neural activation noted in the LS and LHb differed between males and females only in the case of AIS, with a higher degree of LS and LHb activation observed in males. Prior studies in males have reported an increase in Fos expression in the LS and LHb following both AIS and FS (Aloisi, Zimmermann, & Herdegen, 1997; Duncan, Knapp, Johnson, & Breese, 1996). While comparative studies have thus far not addressed differences in the pattern and extent of neural activation in males versus females, previous reports do indicate that female rats also exhibit acute stress mediated activation of the LS and LHb (Aloisi et al., 1997). The LS receives inputs from the mPFC, hippocampus, amygdala, bed nucleus of stria terminalis (BNST) and the entorhinal cortex, and sends outputs to different lateral and medial hypothalamic nuclei and the periaqueductal gray (PAG). The LS also receives dense monoaminergic innervation from stress-responsive midbrain monoaminergic systems (Sheehan, Chambers, & Russell, 2004). The LHb along with the medial habenula and the pineal gland form part of the epithalamus, receiving inputs from the mPFC, basal ganglia, and basal forebrain and sending outputs to the PAG, brainstem, and the monoaminergic systems of the brain (Batalla et al., 2017). Hence, by virtue of connectivity, the LS and LHb serve as putative node structures integrating top-down information from the mPFC and the hippocampus along with information from the hypothalamus, amygdala, and BNST as well as modulatory inputs from monoaminergic systems (Batalla et al., 2017; Sheehan et al., 2004). These structures have been suggested to serve as relay circuitry to hypothalamic nuclei and the PAG as well as the ventral tegmental area, regions involved in executing motivated and behavioral responses to stressor stimuli (Batalla et al., 2017; Sheehan et al., 2004). Our observations that AIS results in a higher degree of neural activation within the LS and LHb in males, raises the intriguing possibility of sexual dimorphism in the extent of recruitment of these node structures by acute stress, suggesting sex differences in the perception and processing of acute stress.

Neural activation within the primary integrator circuit for stress signals, namely the PVN (Radley et al., 2009) and in the fear processing circuitry of the amygdala, namely the CeA and BLA (Babb et al., 2013; Khurana & Devaud, 2007), did not differ across males and females following either AIS or FS. We observed that both AIS and FS resulted in a robust enhancement in the number of Fos positive cells in the PVN and amygdaloid nuclei across both sexes. The PVN is the central brain region that initiates the neuroendocrine response to a stress resulting in the eventual increase in circulating levels of the adrenal glucocorticoid, corticosterone (Radley et al., 2009). The amygdala is known to be activated by stress-induced release of glucocorticoids and is thought to be responsible for the formation of stress-related anxiety (Gehlert et al., 2005; Pith et al., 1995). Together, the PVN and the amygdala are part of a system that help to initiate and maintain a stress response (Ulrich-Lai & Herman, 2009). Although prior studies clearly reveal differences in HPA responsiveness to stress in females (Babb et al., 2013), we do not see this reflected in the degree of Fos expression in the PVN post AIS or FS. Previous reports have shown that restraint stress led to a similar extent of upregulation in Fos mRNA in the PVN of males and female rats (Figueiredo et al., 2002). Our results are consistent with previous reports of acute stress mediated neural activation of the PVN and the amygdala (Babb et al., 2013; Khurana & Devaud, 2007; Radley et al., 2009) and further indicate that at least in the context of AIS and FS no sex differences are observed in the extent and pattern of neural activation in these primary stress-responsive subcortical neural circuits.

Within the brain, there seems to be a functional hierarchy of regions in terms of perceiving and responding to a stress. The subcortical regions of the PVN and amygdala are responsible for initiation and maintenance of a stress response and higher cortical regions like the mPFC and the hippocampus are involved in processing information about the stressor that will help the organism adopt the appropriate coping mechanism and mount a suitable behavioral response (Ulrich-Lai & Herman, 2009). This top-down information is transmitted to nodal points, including the LS and the LHb, which further relay it to subcortical circuitry of the hypothalamus, VTA, and pathways that control autonomic, motor, and nociceptive responses, such as the PAG, with the goal of mounting adaptive responses that promote coping and resilience to stress (Keay & Bandler, 2001; Morilak et al., 2005). The picture that emerges from our results suggest that while the primary initiator circuitry of the PVN and amygdala do not differ in the extent of neural activation evoked by AIS or FS, clear sex differences are noted in the extent and pattern of neural activation within the mPFC and hippocampus, as well as changes within nodal circuits of the LS and LHb. This raises the speculative possibility that sex differences in stress processing may be noted in particular within higher-order cortical stress.
processing neurocircuits rather than in primary initiator circuits, thus altering top-down control of specific aspects of stress responses, such as nociception, anhedonia, fear, and despair.

Further, the differences in neural activation within stress-processing neurocircuitry also exhibit variation based on the nature of the stressor involved. The two models of stress we have used namely AIS and FS, with the former often described as a psychological stressor, and FS which has the component of both active and passive coping strategies, including manifestation of learned helplessness-like behaviors on the second day (Colom-Lapetina et al., 2017). Further, it is important to note that while we subjected animals to a single episode of AIS, the FS paradigm similar to what is used in the modified forced swim test involved exposures to forced swim across two days, hence also bringing in the component of variation in duration of stressor experience. Given FS involves two exposures to swim stress, differences noted in Fos expression patterns could also arise due to sex-dependent differences in habituation to stress. Thus, these two distinct stressors differed both in the nature of threat perception and in the duration of exposure, possibly recruiting distinct components of higher-order stress processing circuitry. However, despite these clear differences in the stressors used we note that for each individual stressor, the pattern and degree of neural activation differs in cortical circuits of the mPFC and hippocampus, but does not vary in the primary initiator subcortical circuits of the PVN and the amygdala, across sexes. We also noted clear sex differences in the degree of neural activation in the LS and LHb only for AIS, but not in the case of FS. It is noteworthy that we observed that sex-dependent patterns of neural activation varied depending on the nature of the stressor. This was further highlighted based on cross-correlation analyses, which clearly showed that following AIS, females show significant correlation of Fos induction across multiple brain regions (Supplementary Figure S1). In striking contrast, males subjected to AIS did not show a similar pattern of correlation of Fos across brain regions, with significance noted only within hippocampal and mPFC subfields (Supplementary Figure S1(a, c)). The stark sex difference in correlation of Fos expression across brain regions noted following AIS, was not observed following FS (Supplementary Figure S1(b,d)). This correlation analysis clearly indicated that the sex-differences in neural activation patterns are more pronounced following AIS. This observation serves to caution against generalizing findings of sex-differences in neural activation across acute stress models and provides impetus for future studies that address the specific stressor contexts in which sex differences in recruitment of stress-processing circuitry are observed.

While our results allow us to assess potential differences in the degree and pattern of neural activation following stress, based on profiling of Fos expression, they do not provide a mechanistic insight into how such sex differences in stress-evoked regulation of Fos expression arise. Although it is most likely that differences in numbers of Fos expressing cells in specific brain regions is reflective of the extent of neural activation within those circuits, it is also important to note that transcriptional regulation of Fos has been shown to involve a role for sex steroids and estrogen/androgen receptors (Duan, Xie, Li, McDougal, & Safe, 2002). Prior results indicate behavioral response to FS do not appear to vary according to stage of estrous cycle (Kokras et al., 2015), although we cannot preclude an influence on Fos expression. As we did not measure circulating hormone levels across the estrous cycle of females used in our studies, either under baseline conditions or following stress, we cannot draw a conclusion about the contribution of circulating sex steroids to the differences noted in stress-evoked Fos expression.

Stress serves as an important risk factor for psychiatric disorders such as anxiety and depression. Several studies clearly indicate sex differences in risk for the development of stress-associated disorders of anxiety, depression, posttraumatic stress disorder, and cardiovascular dysfunction with women exhibiting enhanced risk for these diseases. This raises the possibility that such enhanced risk based on sex may arise due to differences in processing and perception of stress, and in the ability to mount appropriate adaptive, stress-coping, and resilient responses. Sexual dimorphism in the neural activation patterns of different stress responsive regions of the brain could contribute to our understanding of the differential recruitment of these circuits in the male and female brain, thereby allowing us to better understand the sex-dependent bias in the occurrence of stress-associated disorders.

Disclosure statement

No potential conflict of interest was reported by the authors.

References


