Sex-dependent effects of traumatic stress on social behavior and neuronal activation in the prefrontal cortex and amygdala

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Abstract

Social behavior is complex and fundamental, and deficits in social behavior are common pathological features for a variety of psychiatric disorders including anxiety, depression, and posttraumatic stress disorder. Acute stress has a negative impact on social behavior, and these effects may vary based on sex. The aim of this study was to explore the effect of footshock stress on the sociability of male and female C57Bl/6J mice. Animals were divided into two main groups of footshock exposure or context exposure control. Each group had mice that were treated with either the benzodiazepine alprazolam, or vehicle. Neuronal activation during social interaction was assessed using immunohistochemistry against the immediate early gene product cFos. Footshock stress induced a significantly increased latency to approach a social interaction counterpart in both sexes. Stress-induced increases in defensive tail-rattling behavior elicited during the sociability test were sex-dependent and alleviated by alprazolam. Alprazolam also lowered social exploration and neuronal activation in the infralimbic medial prefrontal cortex. Social interaction induced sex-dependent differences in cFos activation in the lateral subdivision of the central nucleus of the amygdala and ventromedial intercalated cell clusters. Overall, our results suggest that acute footshock stress induces alterations in sociability and patterns of cFos activation in a sex-dependent manner.

Introduction

Social behavior is important for survival, and social deficits represent common pathological features for a variety of mental illnesses including social anxiety disorder, depression, and posttraumatic stress disorder [1, 2]. More women than men suffer from these disorders, yet there is a paucity of data on sex differences in social behavior after stress [3]. There are data suggesting that childhood trauma leads to more aggressive behavior in men and more social withdrawal and avoidance in women [4], and women with social anxiety disorder report greater clinical severity, which may be associated with stressful life experiences [5]. Therefore, understanding sex differences in the effects of traumatic stress on social behavior, as well as the underlying neural substrates that potentially control this behavior has important translational relevance.

Animal models of stress and trauma show alterations in many aspects of behavior [6]. In general, both acute and chronic stress have been found to lead to social fear and withdrawal in rodents [7]. One common model of traumatic stress in rodents is footshock exposure [8]. However, there is a lack of data investigating whether footshock stress influences mouse social behavior in a sex-dependent manner.

It is also unclear whether there are sex differences in activation of brain regions following social encounters. Several lines of evidence suggest that neural activity in the medial prefrontal cortex (mPFC) is important for social behavior and subregions of the mPFC are differentially activated following social interaction. Neurons in the infralimbic cortex (IL) are preferentially activated in response to social cues, compared to neurons in the prelimbic cortex (PL) [9], and a social neural ensemble within the IL may contribute to the social buffering of fear after fear conditioning [10].

Several subnuclei of the amygdala have also been implicated in social behavior. The role of the basolateral amygdala in social interaction following different stress paradigms has been well-established [12-14]. For example, activation of the basolateral amygdala leads to reduced social interaction in a social interaction test [11]. The medial nucleus of the amygdala (MeA) is also involved in both social behaviors and responses to stressors [15]. cFos expression in the intercalated nucleus of the amygdala (ITC) is increased during social buffering in rats [16], and social interaction is impaired in mice with altered migration and differentiation of ITCs [17, 18]. Among the amygdala regions implicated in social behavior, the central nucleus of the amygdala (CeA) is relatively unexplored. There is recent evidence, however, that CeA circuits may be linked to sociability, and some manipulations of CeA activity impact social behavior [18-21].

Thus, multiple brain regions are involved in social cognition [15, 16], but the differences in social approach or avoidance behavior following acute stress are still poorly understood. In the current study, our goal was to assess the extent to which there are sex differences in social interaction, using social interaction tests that eliminate the possibility of direct physical aggression. We hypothesized that electric footshock stress would negatively affect sociability, and that those changes might depend on sex and be reversible with the fast-acting benzodiazepine-alprazolam. Therefore, we performed social interaction tests 24 hours after two days of footshock stress. Additionally, we aimed to determine patterns of neuronal activation in the IL,

CeA, MeA, and ventral ITC associated with social behaviors using expression of the immediate early gene cFos.

Materials and methods

Animals

2–4-month-old male and female C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, Stock No: 000664) and housed on a 12 h light/dark cycle with *ad libitum* access to water and chow under standard laboratory conditions. Mice were individually housed for 7 days before the start of and all throughout the experiments. Experiments were performed during the light phase. All animal procedures were performed in accordance with institutional guidelines and were approved by the Institutional Animal Care & Use Committee of Tulane University. Unfamiliar strain-, sex- and age-matched mice were used as the passively interacting counterparts (stimulus mice) during social interaction tests.

Footshock stress exposure

Footshock exposure or control context exposure was conducted in standard mouse operant conditioning chambers (ENV-307W, Med Associates, Inc., St. Albans, VT) enclosed within sound- and light-attenuating cubicles (ENV-022MD, Med Associates, Inc., St. Albans, VT). The chambers were connected to a computer through an interface and controlled by MED-PC software. The chamber was equipped with a grid floor and a house light, which was cleaned using 70% ethanol.

Seven days after single housing, mice underwent footshock exposure for two consecutive days. Each of the shock sessions included five 1 s, 0.9 mA footshocks presented with a 120 s average pseudorandom intertrial interval (range 90−150 s), totaling 800 s in the chamber. Mice in the control group were exposed to the same chambers for the same period but did not experience footshock (Fig. 1A).

Social interaction test

The day after footshock exposure, the mice underwent the social interaction test in a square 46 x 46 X 38 cm arena constructed from sheets of white plexiglass (Fig. 1B). Behavioral videos were recorded using a digital camera (Allied Vision “Pike” camera, Germany) and Plexon Studio tracking software (Plexon, Dallas, TX). Tests were conducted under dim (10.6 lux) white fluorescent lighting. Stimulus mice were single housed for 3 days before tests. Each of the stimulus mice interacted with three experimental mice with at least 30 min between tests. Experimental mice were perfused 90 min after the test to assess cFos expression.

An indirect social interaction method was chosen to avoid physical aggression between male mice. For the first 3 min, mice were allowed to explore the open arena with two rectangular (15 X 5 X 6 cm) metallic mesh boxes located in opposing corners 5 cm away from the walls. After

115 the initial exploration, an unfamiliar, untreated stimulus mouse was put underneath one of the
116 boxes. Behavior was recorded for an additional 5 minutes, and sociability was scored using time
117 spent sniffing the mesh box containing the stimulus mouse as a percentage of total box
118 interaction time (mouse preference, %), the latency to approach the stimulus mouse, and the
119 number of defensive tail rattles. Total exploration of the mesh boxes was also scored in seconds
120 to assess general activity. All behavioral measurements were scored by an observer blinded to
121 condition. Consistent with other social interaction scoring protocols [22], sniffing directed to the
122 upper and top part of the mesh boxes, sniffing of feces, bar biting and circulating around the
123 corral without sniffing, were not scored as social interaction.

124

125 *Alprazolam treatment*

126 Alprazolam (Sigma-Aldrich, St. Louis, MO) was dissolved in a drop of Tween 80 (Merck,
127 Germany) and saline was added to make a final dose of 0.25 mg/kg. This dose was shown to
128 have anxiolytic effects [23] with minimal motor impairment in C57BL/6J mice [24]. Tween 80 +
129 saline solution was used for vehicle injections. Solutions were administered at 10 ml/kg volume,
130 intraperitoneally, 30 min before social interaction tests.

131

132 *Histology*

133 Following testing, mice were anesthetized with 2,2,2-tribromoethanol (240 mg/kg, ip, Sigma)
134 and subsequently transcardially perfused with 4% paraformaldehyde in phosphate-buffered
135 saline (PBS). cFos expression was assessed in mice that were perfused 90 min after the social
136 interaction test. Fixed brains were cut on a compresstome vibrating microtome (Precisionary,
137 Greenville, NC) in 80 µm coronal slices.

138 Antibody staining was performed on free-floating tissue sections. After 3 x 10 min washes with
139 0.5% PBST slices were put in 5% donkey serum for 2 hours. Sections were then incubated
140 overnight in primary antibodies at 4°C. On the next day sections were washed in 0.5% PBST (3
141 X 10 min), and then went through a 2 hr incubation with secondary antibodies at 4°C. After 3 x
142 10 min washes in PBS slices were mounted with mounting medium with DAPI (Biotium,
143 Fremont, CA). The primary antibody was rabbit anti-cFos (1:1500; 226 003, Synaptic Systems,
144 Germany), and the secondary antibody was donkey anti-rabbit AlexaFluor 488 (1:500; A-21206,
145 Thermo Fisher Scientific, Waltham, MA) cFos.

146 Images were obtained using an AxioScan.Z1 slide-scanning microscope (Zeiss, Germany) and
147 a Nikon A1 Confocal microscope (Nikon, Japan). cFos-positive nuclei were quantified using Fiji
148 ImageJ software (NIH, Bethesda, USA), and averaged for each animal. A blinded observer
149 quantified c-Fos expression in 2-5 slices per structure per mouse.

150

151 *Statistical analysis*

152 Data were analyzed using Prism 9 (GraphPad Software, San Diego, CA). The definition of
153 statistical significance was $P \leq 0.05$. Two male mice that exhibited no interaction with either
154 mesh box during the social interaction test were excluded from analysis. To assess the

interaction of factors, a 2- or 3-way ANOVA was used. If a significant effect was detected, Sidak's multiple comparisons test was used, because it assumes independent comparisons and has more power than the Bonferroni method. Correlations between cFos expression and behavior were analyzed using pooled data from stressed and control mice that were treated with alprazolam or vehicle. Using the Kolmogorov-Smirnov test, the data were determined to be non-parametric, so Spearman's correlation coefficient was used for this analysis. For the sake of clarity, we report the results of the interaction tests, the significant simple main effects, and the significant post-hoc tests in the main text. The results of all tests are reported in Table 1. All statistical tests were two-tailed.

Results

Effects of footshock stress on social behavior

Following two days of footshock stress or context exposure, male (N = 13 control, N = 11 stress) and female (N = 12 control, N = 13 stress) mice were subjected to a social interaction test designed to measure sociability (Fig. 1A, B). A two-way ANOVA revealed that there was no statistically significant interaction between the effects of sex and stress on preference for the social stimulus (Fig. 1C; sex X stress, $F_{(1,45)} = 1.08$, $p = 0.31$). Simple main effects analysis showed that neither sex nor stress had a statistically significant effect on social preference (Fig. 1C; Table 1). There was no significant interaction between stress and sex on the latency to approach the social stimulus (Fig. 1D; 2-way ANOVA, sex X stress, $F_{(1,45)} = 0.57$, $p = 0.45$); however, simple main effects analysis showed that stress had a significant effect (stress effect, $F_{(1,45)} = 4.65$, $p = 0.04$). Further, stressed males displayed significantly more tail-rattling behavior than control males during the social interaction test, whereas females in both groups displayed equivalent levels of tail rattling (Fig. 1E; 2-way ANOVA, stress X sex, $F_{(1,45)} = 4.04$, $p = 0.05$; Sidak's multiple comparisons test, stressed versus control males, $p = 0.03$). Exploratory behavior, measured as total interaction time, was unaffected by footshock stress (Fig. 1F; 2-way ANOVA, sex X stress, $F_{(1,45)} = 0.1698$, $p = 0.68$).

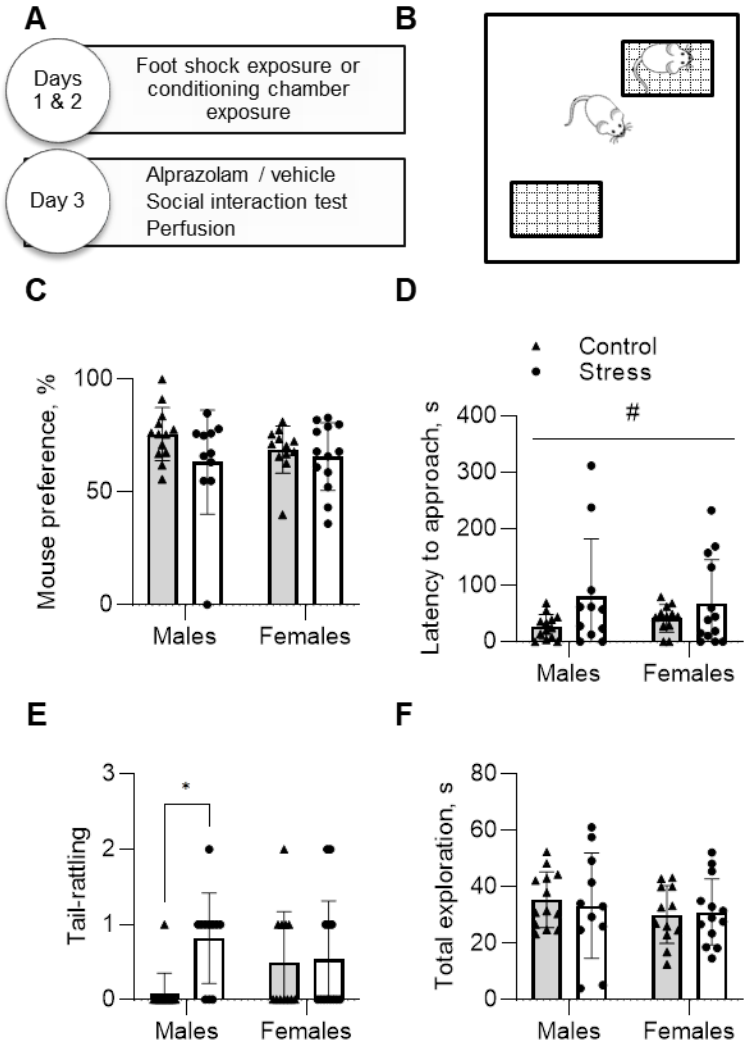

Fig. 1. Effects of acute stress on social interaction. **A.** Experimental timeline. **B.** Schematic of the social interaction arena. **C.** Preference for the social stimulus was not significantly altered by stress in either males or females during the social interaction test. **D.** Stress significantly increased the latency to approach the social stimulus in both males and females. **E.** Total number of tail rattles. Defensive tail-rattling behavior was differently expressed in males and females. **F.** Exploratory behavior was not significantly different in males or females and was not altered by stress. Data are presented as means ± SEM. *p<0.05, post-hoc tests. #p<0.05, stress effect.

Effects of alprazolam

Next, we evaluated the effect of alprazolam on shock-induced changes in social interaction (Control vehicle: N = 13 male, 12 female; control alprazolam: N = 5 male, 5 female; stress vehicle: N = 11 male, 13 female; stress alprazolam: N = 5 male, 5 female). A three-way ANOVA revealed that there was no statistically significant interaction between the effects of sex, stress, and alprazolam on preference for the social stimulus (Fig. 2A; drug X sex X stress, $F_{(1,63)}$ = 0.32,

p = 0.57). A simple main effects analysis showed that alprazolam significantly lowered mouse preference (Fig. 2A; drug effect, $F_{(1,63)}$ = 4.28, p = 0.05). Alprazolam had no significant effect on the latency to approach the stimulus mouse (Fig. 2B; 3-way ANOVA, drug X sex X stress, $F_{(1,63)}$ = 0.41, p = 0.52). A three-way ANOVA was performed to analyze the effect of sex, stress, and alprazolam on tail rattling behavior. This analysis revealed that there was no interaction between these effects (Fig. 2C; 3-way ANOVA, drug X sex X stress, $F_{(1,63)}$ = 2.55, p = 0.12). A simple main effects analysis showed that tail rattling was significantly reduced following alprazolam treatment (drug effect, $F_{(1,63)}$ = 10.5, p = 0.002). There were significant differences between alprazolam- and vehicle-treated stressed males (Fig. 2C; Sidak's multiple comparisons test, p = 0.04), and between stressed and control groups of vehicle-injected male mice (Fig. 2C; Sidak's multiple comparisons test, p = 0.01). There was no significant interaction between the effects of stress and alprazolam on total exploration time (Fig. 2D; 3-way ANOVA, drug X sex X stress, $F_{(1,63)}$ = 0.01, p = 0.91).

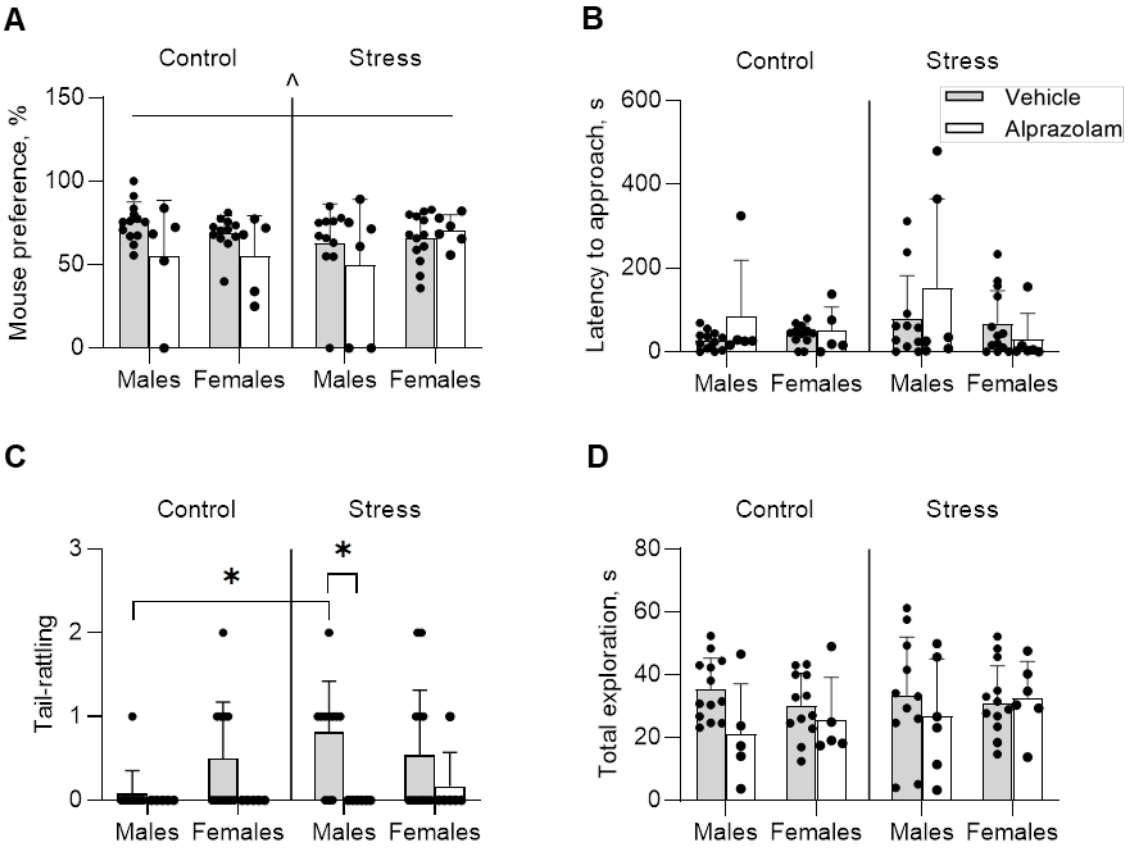

Fig. 2. Effects of alprazolam on social and exploratory behaviors of stressed mice. **A.** Alprazolam reduced preference for the social stimulus. **B.** Latency to approach was not significantly affected by alprazolam. **C.** Tail rattling was increased by stress in male mice and was significantly reduced by alprazolam in both sexes. **D.** Exploratory behavior was not significantly altered by stress or drug treatment. Data are presented as means ± SEM. *p<0.05 post hoc tests. ^p<0.05, drug effect.

cFos expression analysis

222
223 We next quantified expression of cFos in several brain areas involved in the regulation of social
224 behavior (Fig. 3 A, B). A three-way ANOVA was performed to test for the effects of sex, stress,
225 and alprazolam treatment on cFos expression in the IL, CeA, ventral ITC, and MeA (N = 11
226 control vehicle (3 male, 8 female), N = 10 control alprazolam (5 male, 5 female), N = 14 stress
227 vehicle (5 male, 9 female), N = 8 stress alprazolam (4 male, 4 female)). There was no significant
228 interaction between the effects of these variables on cFos expression in any of the brain areas
229 analyzed (see Table 1). A main effects analysis showed that alprazolam treatment significantly
230 reduced cFos expression in the IL (Fig. 3C; drug effect, $F_{(1,35)} = 10.5$, $p = 0.003$). Control
231 females injected with alprazolam had significantly fewer cFos+ cells in the IL compared to
232 vehicle-injected female controls (Sidak's multiple comparisons test, $p = 0.02$). There were no
233 significant simple main effects of sex, stress, or drug on cFos expression in the capsular
234 subdivision of the CeA (Fig. 3D), the medial subdivision of the CeA (Fig. 3E), or in the medial
235 amygdala (Fig. 3H). A simple main effect analysis showed that sex had a significant effect on
236 cFos expression in the lateral CeA, with greater expression levels in males compared to
237 females (Fig. 3F; sex effect, $F_{(1,28)} = 5.06$, $p = 0.03$). There was also a significant effect of sex on
238 cFos expression in the ventromedial intercalated nucleus of amygdala, with greater expression
239 levels in males (Fig. 3G; sex effect, $F_{(1,28)} = 7.05$, $p = 0.01$)

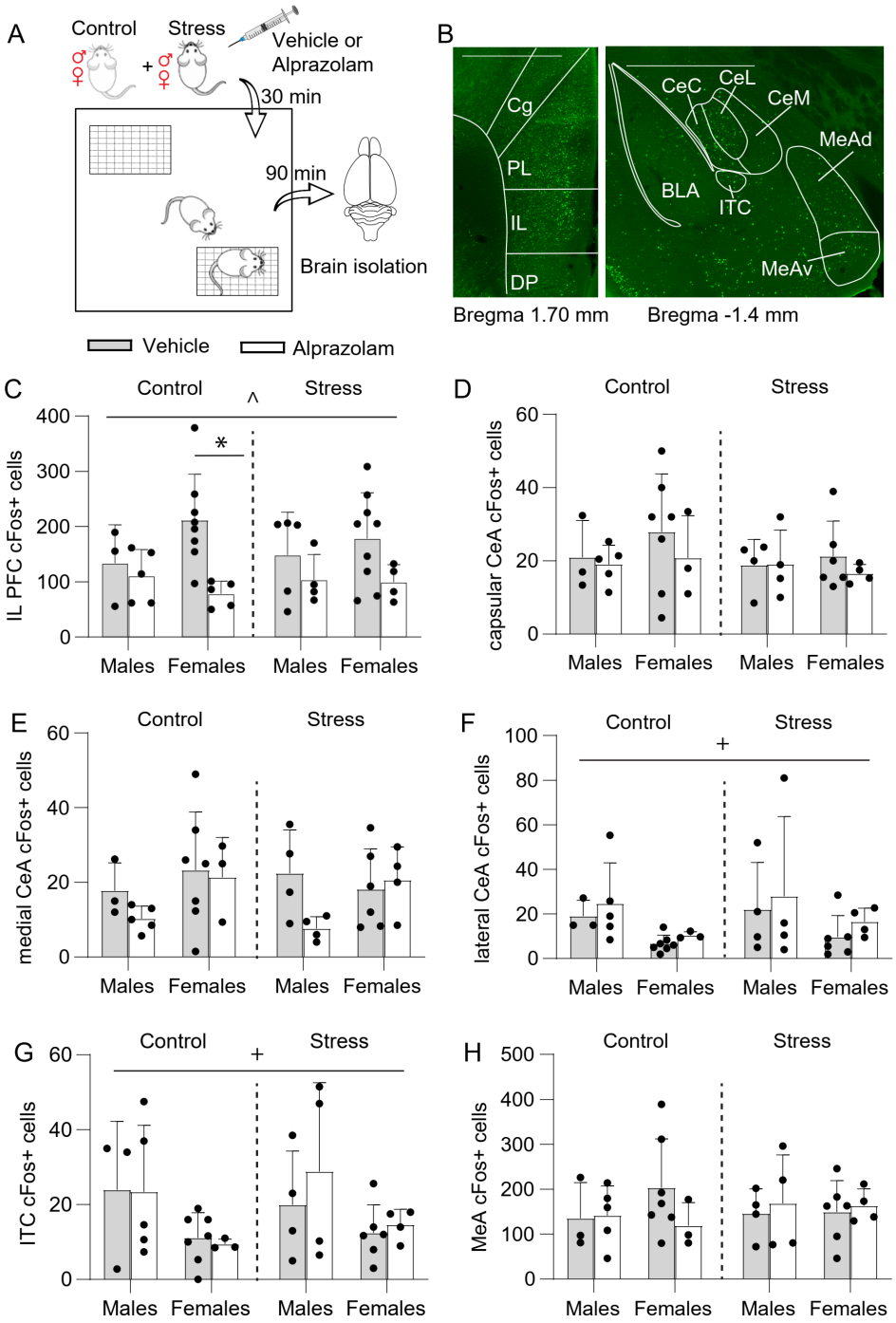

Fig. 3. cFos expression patterns following social interaction. **A.** Schematic of the experiment. **B.** Representative images of cFos staining results in mPFC (A) and amygdala (B). IL = infralimbic mPFC, CeC = capsular CeA, CeL = lateral CeA, CeM = medial CeA, ITC = ventromedial intercalated nucleus of amygdala, MEAd = dorsal MEA, MEAv = ventral MEA. Scale: 1000 µm. **C-H.** Average number of cFos+ cells / mouse. **C.** Alprazolam reduced cFos expression in the IL. **D-E.** c-Fos expression in the capsular and medial CeA was unaffected by stress, sex, or drug. **F-G.** Expression levels in the lateral CeA and ventral ITCs were greater in males. **H.** cFos

expression in the MeA was unaffected by stress, sex, or drug. Data are presented as means ± SEM. *p<0.05 post hoc tests. ^p<0.005, drug effect. ^{+}p<0.05, effect of sex.

Correlations between cFos levels and behavior following the social interaction test

Spearman's correlation coefficient was used to assess the relationships between behavioral variables, and between behavioral variables and cFos expression levels (Figure 4 and Table 1). These correlation analyses included the data from mice from all groups used in this study. More socially active mice demonstrated less neuronal activation in the CeA (r = -0.532, p = 0.001), particularly in the capsular (CeC, r = -0.434, p = 0.008) and lateral (r = -0.516, p = 0.001) subdivisions, as well as in the ventromedial ITC (r = -0.421, p = 0.011).

Latency to approach the social stimulus positively correlated with the number of cFos+ cells in the CEA (r = 0.39, p = 0.019), in particular in the CeC (r = 0.346, p = 0.039). Total exploration time negatively correlated with the number of cFos+ cells in the lateral CeA (r = -0.365, p = 0.028) and positively correlated with IL mPFC activation (r = 0.357, p = 0.019). Tail rattling behavior was most strongly correlated with cFos activation in the medial CEA, although this did not reach statistical significance (r = 0.313, p = 0.063).

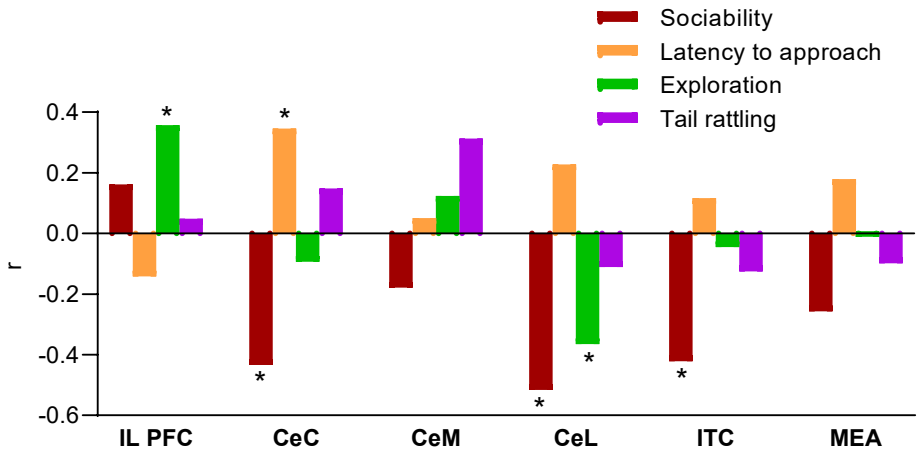

Fig 4. Correlations between the number of cFos positive cells and behavioral parameters. Spearman's correlation coefficients (r) between behavioral parameters (sociability, latency to approach, exploration, tail rattling) and cFos expression in IL, CeA subdivisions, ITC and MeA in mice from all groups used in this study. Spearman's correlation coefficient represented with * indicating significant correlations (p<0.05). CeC = capsular CeA, CeM = medial CeA, CeL = lateral CeA, ITC = ventromedial intercalated nucleus of amygdala.

Discussion

Exposure to footshock is a commonly used model of traumatic stress to assay core features of stress disorders such as social withdrawal [25]; however, sociability after footshock stress has

been investigated predominantly in male rodents. In the current study, several measurements of social interaction behavior were taken in male and female mice to investigate potential sex differences in the impact of stress on sociability (Figure 1) and neuronal activation in the IL, CeA, and MeA (Figure 3). The main impact on social interaction was that mice of both sexes exhibited a significantly increased latency to approach the social stimulus following footshock stress (Figure 1). Previous research has shown that footshock exposure increases anxiety-like behavior suggesting that the observed changes in social interaction might be part of an enhanced general anxiety-like phenotype that emerges following footshock stress [7]. Therefore, given its known anxiolytic effects, we expected alprazolam to reverse stress-induced changes in latency to approach [7, 26]. Although it is possible that a higher dose of alprazolam would lead to a reversal of increased approach latency after stress, doses higher than that used in the current study have deleterious locomotor effects [26, 27]. An alternative hypothesis is that the latency to approach a conspecific is more of a social motivation measure [28] that is separate from mouse preference, the main measure of sociability used here. Interestingly, the main effect of alprazolam on mouse preference may be evidence that social interaction itself is anxiogenic, even under control conditions [29]. This is further supported by the negative correlation between sociability and cFos expression in the CeA (Figure 4).

Our findings suggest that tail rattling behavior during social interaction is sex-dependent, which is affected by stress specifically in males (Figure 1) and diminished by alprazolam (Figure 2). Tail rattling is elicited due to territorial aggression in male mice [31, 32], and it has been suggested that it can be a measurement of threat-induced defensive aggression [32]. Our results are therefore comparable to other mouse sociability studies in which footshock stress produced an enhancement of aggression in male mice [10]. Interestingly, female mice exhibit more tail rattling than males during fear conditioning [33], suggesting that sex differences in tail rattling behavior are both stress- and context-dependent. The significant reduction of tail rattling by alprazolam further supports the link between this behavior and negative valence states. Overall, the observed sex differences reinforce the idea that sex is a crucial factor that should be considered in stress-related studies.

To the best of our knowledge, this is the first study reporting sex-dependent differential expression profile of cFos in the CeA after social interaction. Our results show that cFos expression in the lateral CeA and ITC is higher in males compared to females after social interaction. Given the known role of these amygdala regions in mediating threat responses [17, 34], these data suggest that social interaction may induce a higher level of defensiveness in male mice. Consistent with this hypothesis, previous work has shown that CeA neuronal activation is associated with male mouse aggression during social interaction in the resident intruder assay [35, 36]. These findings emphasize the need to study sex differences while deciphering the relationship between stress and social behaviors. Further investigations should be conducted into sex differences in different neuronal populations of CeA, expressing molecular markers such as somatostatin or corticotropin-releasing factor. This is especially relevant given that there is a greater number of corticotropin-releasing factor receptor 1-containing neurons in the male compared to female CeA, and they have different neuronal excitability in response to corticotropin-releasing factor [37].

Social investigation and IL PFC cFos levels, but not total exploration time, were decreased due to alprazolam treatment both in stressed and control mice. The reduced social interaction effect of acute benzodiazepine treatment is consistent with previous findings [26], as well as attenuation of cFos expression during novelty stress exposure in the mPFC, but not in the amygdala [30]. cFos expression in IL mPFC in our study was not significantly affected by stress and only correlated with exploratory behavior, but not social investigation. Thus, although the IL has been shown to be activated in response to a social cue [9], the IL mPFC does not seem to modulate the interaction between traumatic stress and sociability in mice.

The MeA has been linked to a wide variety of social behaviors, such as aggression, mating, and parenting [38]. Here, we find that MeA cFos expression induced by the social interaction test is not affected by sex, stress, or alprazolam. Prior studies have demonstrated that socially defeated females housed with aggressive male residents exhibit increased cFos activation in the medial amygdala [39], and the MeA is more responsive to aggressive than to benign social interaction [40]. We designed our experiments to exclude the possibility of aggressive interactions, therefore free interactions between the experimental and stimulus mice were not possible. Future studies could investigate the effects of stress and sex on MeA activation in free interaction paradigms.

The social consequences of stress have been extensively studied using the social defeat stress model that entails exposure to emotional / psychological stress and leads to depression-related outcomes [7]. After the social defeat procedure, most mice develop a decreased drive to approach and interact with the social target [41]; however, social defeat stress is difficult to achieve in female mice. Acute stress paradigms utlizing footshock facilitate investigations into sex differences in social behavior following trauma. Future work should define the optimal conditions, such as footshock intensity or lighting conditions, that influence sociability after stress. It could be especially valuable to develop paradigms that stratify mice as resilient and susceptible to further validate acute footshock stress as a tool for PTSD research.

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The authors declare no competing interests.

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Formal analysis—MD, CDB, JPF
Funding acquisition—JPF
Investigation—MD, CDB, KW, AR, SB, EB, AD

Methodology—MD, CDB, JPF
Project Administration / Supervision—MD, JPF
Resources—JPF
Visualization—MD, CDB, JPF
Writing, original draft—MD, JPF
Writing, review & editing—MD, JPF, CDB, LSO, KW

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481 **Figure legends**

482 **Fig. 1.** Effects of acute stress on social interaction. **A.** Experimental timeline. **B.**
483 Schematic of the social interaction arena. **C.** Preference for the social stimulus was not
484 significantly altered by stress in either males or females during the social interaction test. **D.**
485 Stress significantly increased the latency to approach the social stimulus in both males and
486 females. **E.** Total number of tail rattles. Defensive tail-rattling behavior was differently expressed
487 in males and females. **F.** Exploratory behavior was not significantly different in males or females
488 and was not altered by stress. Data are presented as means ± SEM. *p<0.05, post-hoc tests.
489 #p<0.05, stress effect.
490 **Fig. 2.** Effects of alprazolam on social and exploratory behaviors of stressed mice. **A.**
491 Alprazolam reduced preference for the social stimulus. **B.** Latency to approach was not
492 significantly affected by alprazolam. **C.** Tail rattling was increased by stress in male mice and
493 was significantly reduced by alprazolam in both sexes. **D.** Exploratory behavior was not
494 significantly altered by stress or drug treatment. Data are presented as means ± SEM. *p<0.05
495 post hoc tests. ^p<0.05, drug effect.
496 **Fig. 3.** cFos expression patterns following social interaction. **A.** Schematic of the
497 experiment. **B.** Representative images of cFos staining results in mPFC (A) and amygdala (B).
498 IL = infralimbic mPFC, CeC = capsular CeA, CeL = lateral CeA, CeM = medial CeA, ITC =
499 ventromedial intercalated nucleus of amygdala, MEAd = dorsal MEA, MEAv = ventral MEA.
500 Scale: 1000 µm. **C-H.** Average number of cFos+ cells / mouse. **C.** Alprazolam reduced cFos
501 expression in the IL. **D-E.** c-Fos expression in the capsular and medial CeA was unaffected by
502 stress, sex, or drug. **F-G.** Expression levels in the lateral CeA and ventral ITCs were greater in
503 males. **H.** cFos expression in the MeA was unaffected by stress, sex, or drug. Data are
504 presented as means ± SEM. *p<0.05 post hoc tests. ^p<0.005, drug effect. ^{+}p<0.05, effect of
505 sex.

506 **Fig 4. Correlations between the number of cFos positive cells and behavioral**
507 **parameters**. Spearman's correlation coefficients (r) between behavioral parameters (sociability,
508 latency to approach, exploration, tail rattling) and cFos expression in IL, CeA subdivisions, ITC
509 and MeA in mice from all groups used in this study. Spearman's correlation coefficient
510 represented with * indicating significant correlations (p<0.05). CeC = capsular CeA, CeM =
511 medial CeA, CeL = lateral CeA, ITC = ventromedial intercalated nucleus of amygdala.

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523 **Table 1: Statistical data**

Source	Sum of squares	*DFn*	*DFd*	*F*	*p*
Effects of foot shock stress x sex: 2-way ANOVA					
Fig. 1C Sociability (Mouse preference, %)					
Stress*Sex	260.7	1	45	1.08	0.31
Stress	725.8	1	45	2.99	0.09
Sex	61.21	1	45	0.25	0.62
Fig. 1D Latency to approach					
Stress*Sex	2387	1	45	0.57	0.45
Stress	19475	1	45	4.65	0.04*
Sex	12.95	1	45	0.003	0.96
Fig. 1E Tail rattling					
Stress*Sex	1.505	1	45	4.04	0.05
Stress	1.853	1	45	4.97	0.03*
Sex	0.063	1	45	0.17	0.68
Fig. 1F Exploration					
Stress*Sex	28.15	1	45	0.17	0.68
Stress	4.715	1	45	0.03	0.87
Sex	167.7	1	45	1.01	0.32
Effects of alprazolam x foot shock stress x sex: 3-way ANOVA					
Fig. 2A Sociability (Mouse preference, %)					
Alprazolam*Stress*Sex	131.1	1	63	0.32	0.57
Alprazolam*Stress	576.0	1	63	1.41	0.24
Alprazolam*Sex	607.5	1	63	1.49	0.23
Stress*Sex	864.5	1	63	2.11	0.15
Alprazolam	1711	1	63	4.18	0.05*
Stress	35.47	1	63	0.09	0.77
Sex	254.4	1	63	0.62	0.43
Fig. 2B Latency to approach					
Alprazolam*Stress*Sex	3406	1	63	0.41	0.52
Alprazolam*Stress	935.1	1	63	0.11	0.74
Alprazolam*Sex	23991	1	63	2.91	0.09
Stress*Sex	12700	1	63	1.54	0.22
Alprazolam	9453	1	63	1.15	0.29
Stress	15524	1	63	1.89	0.18
Sex	22768	1	63	2.76	0.10

Fig. 2C Tail rattling					
Alprazolam*Stress*Sex	0.7122	1	63	2.55	0.12
Alprazolam*Stress	0.354	1	63	1.27	0.27
Alprazolam*Sex	0.0005	1	63	0.002	0.97
Stress*Sex	0.271	1	63	0.97	0.33
Alprazolam	2.941	1	63	10.5	0.002*
Stress	0.844	1	63	3.02	0.09
Sex	0.090	1	63	0.32	0.57
Fig. 2D Exploration					
Alprazolam*Stress*Sex	2.603	1	63	0.01	0.91
Alprazolam*Stress	178.9	1	63	0.98	0.33
Alprazolam*Sex	307.8	1	63	1.68	0.20
Stress*Sex	18.37	1	63	0.10	0.75
Alprazolam	529.0	1	63	2.89	0.09
Stress	120.2	1	63	0.66	0.42
Sex	9.883	1	63	0.05	0.82
Effects of alprazolam x foot shock stress x sex on c-Fos: 3-way ANOVA					
Fig. 3C IL PFC					
Alprazolam*Stress*Sex	3452	1	35	0.77	0.39
Alprazolam*Stress	624.4	1	35	0.14	0.71
Alprazolam*Sex	12560	1	35	2.79	0.10
Stress*Sex	235.3	1	35	0.05	0.82
Alprazolam	47156	1	35	10.5	0.003*
Stress	8.845	1	35	0.002	0.97
Sex	3063	1	35	0.68	0.42
Fig. 3D Capsular CeA					
Alprazolam*Stress*Sex	0.018	1	28	0.0002	0.99
Alprazolam*Stress	10.52	1	28	0.10	0.76
Alprazolam*Sex	53.89	1	28	0.51	0.48
Stress*Sex	41.38	1	28	0.39	0.54
Alprazolam	94.65	1	28	0.89	0.35
Stress	89.04	1	28	0.84	0.37
Sex	39.09	1	28	0.37	0.55
Fig. 3E Medial CeA					
Alprazolam*Stress*Sex	69.77	1	28	0.63	0.43
Alprazolam*Stress	4.391	1	28	0.04	0.84
Alprazolam*Sex	269.6	1	28	2.44	0.13
Stress*Sex	32.65	1	28	0.30	0.59
Alprazolam	244.7	1	28	2.21	0.15

Stress	7.754	1	28	0.07	0.79
Sex	334.4	1	28	3.02	0.09
Fig. 3F Lateral CeA					
Alprazolam*Stress*Sex	3.556	1	28	0.01	0.91
Alprazolam*Stress	6.192	1	28	0.02	0.88
Alprazolam*Sex	0.434	1	28	0.002	0.97
Stress*Sex	4.206	1	28	0.02	0.90
Alprazolam	253.1	1	28	0.97	0.33
Stress	118.2	1	28	0.45	0.51
Sex	1318	1	28	5.06	0.03*
Fig. 3G ITC					
Alprazolam*Stress*Sex	15.42	1	28	0.09	0.77
Alprazolam*Stress	93.54	1	28	0.54	0.47
Alprazolam*Sex	32.88	1	28	0.19	0.67
Stress*Sex	13.84	1	28	0.08	0.78
Alprazolam	41.30	1	28	0.24	0.63
Stress	31.62	1	28	0.18	0.67
Sex	1221	1	28	7.05	0.01*
Fig. 3H MeA					
Alprazolam*Stress*Sex	3589	1	28	0.56	0.46
Alprazolam*Stress	6786	1	28	1.06	0.31
Alprazolam*Sex	5170	1	28	0.81	0.38
Stress*Sex	1150	1	28	0.18	0.67
Alprazolam	903.7	1	28	0.14	0.71
Stress	431.3	1	28	0.07	0.80
Sex	969.6	1	28	0.15	0.70

Correlations (Spearman's coefficient)

		Sociability	Exploration	Tail-rattling	Latency to approach
Sociability	r	1.000	**0.499****	0.104	**-0.514****
	p		<0.001	0.389	<0.001
Exploration	r	**0.499****	1.000	0.210	**-0.386****
	p	<0.001		0.079	<0.001
Tail-rattling	r	0.104	0.210	1.000	0.170
	p	0.389	0.079		0.157
Latency to approach	r	**-0.514****	**-0.386****	0.170	1.000
	p	<0.001	0.001	0.157	
IL PFC c-Fos	r	0.162	**0.357***	0.049	-0.142

	p	0.299	0.019	0.754	0.364
Total CEA c-Fos	r	**-0.532****	-0.227	0.165	**0.390***
	p	0.001	0.184	0.337	0.019
Capsular CEA c-Fos	r	**-0.434****	-0.093	0.148	**0.346***
	p	0.008	0.589	0.390	0.039
Lateral CEA c-Fos	r	**-0.516****	**-0.365***	-0.111	0.228
	p	0.001	0.028	0.520	0.182
Medial CEA c-Fos	r	-0.179	0.124	0.313	0.051
	p	0.296	0.473	0.063	0.766
ITC c-Fos	r	**-0.421***	-0.045	-0.125	0.117
	p	0.011	0.795	0.468	0.498
Total MEA c-Fos	r	-0.257	0.007	-0.098	0.179
	p	0.131	0.968	0.571	0.295

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