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Astrocyte focal adhesion kinase reduces passive stress coping by inhibiting ciliary neurotrophic factor only in female mice

Cuihong Jia [*], W. Drew Gill, Chiharu Lovins, Russell W. Brown [1], Theo Hagg [1]

Department of Biomedical Sciences, Quillen College of Medicine, East Tennessee State University, Johnson City, TN, 37614, USA

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ABSTRACT

Astrocytes have been implicated in stress responses and produce ciliary neurotrophic factor (CNTF), which we have shown in the mouse medial amygdala (MeA) to promote passive stress coping response only in females. Pharmacological inhibition of focal adhesion kinase (FAK) upregulates CNTF expression. Here, we found that inducible knockout of FAK in astrocytes or systemic treatment with an FAK inhibitor increased passive coping behavior, i.e., immobility, in an acute forced swim stress test in female, but not male, mice. Strikingly, four weeks of chronic unpredictable stress (CUS) did not further increase passive coping in female astrocytic FAK knockout mice, whereas it exacerbated it in female wildtype mice and male mice of both genotypes. These data suggest that astrocyte FAK inhibition is required for chronic stress-induced passive coping in females. Indeed, CUS reduced phospho-FAK and increased CNTF in the female MeA. Progesterone treatment after ovariectomy activated amygdala FAK and alleviated ovariectomy-induced passive coping in wildtype, but not astrocytic FAK knockout females. This suggests that progesterone-mediated activation of FAK in astrocytes reduces female stress responses. Finally, astrocytic FAK knockout or FAK inhibitor treatment increased CNTF expression in the MeA of both sexes, although not in the hippocampus. As mentioned, MeA CNTF promotes stress responses only in females, which may explain the female-specific role of astrocytic FAK inhibition. Together, this study reveals a novel female-specific progesterone-astrocytic FAK pathway that counteracts CNTF-mediated stress responses and points to opportunities for developing treatments for stress-related disorders in women.

1. Introduction

Stress responses help to maintain physiological homeostasis and function. Excessive or impaired stress responses are linked to a range of mental health disorders, including depression and post-traumatic stress disorder (Deussing and Chen, 2018; Godoy et al., 2018). Women are highly susceptible to these disorders, possibly due to more robust stress responses (Chrousos, 2009; McEwen, 2007; Rincon-Cortes et al., 2019), but the underlying mechanisms are not understood well. The acquired immobility response in rodents during an inescapable stressor, including forced swimming, has recently been recommended as an indicator of passive coping or adaptive behavior (Commons et al., 2017; de Kloet and Molendijk, 2016; Molendijk and de Kloet, 2015). Female rodents have a more robust passive coping response than males (Jia et al., 2019a; Kokras and Dalla, 2014; Xing et al., 2013). Thus, identifying the sex-specific mechanisms underlying adaptive passive coping behavior may provide novel molecular targets for developing treatments for stress-related mental disorders, perhaps specifically for women or men.

Multiple brain areas and cell types, including the amygdala and astrocytes, are involved in stress responses (Deussing and Chen, 2018; Jia et al., 2019a; Jia et al., 2022a; Molendijk and de Kloet, 2019; Molendijk and de Kloet, 2021; Murphy-Royal et al., 2019). CNTF is a member of the interleukin-6 (IL-6) family of cytokines and is almost exclusively expressed in the nervous system (Stockli et al., 1989). In the brain, CNTF is produced by astrocytes (Yang et al., 2008). Brain injury upregulates CNTF, which promotes neurogenesis (Kang et al., 2013) and neuronal survival (Hagg et al., 1992; Hagg and Varon, 1993; Kang et al., 2013). CNTF also enhances cognitive and memory function in rodents (Chohan et al., 2011; Garcia et al., 2010). FAK is a non-receptor tyrosine kinase and a major intracellular transducer of integrin signaling that regulates cell shape, adhesion, and gene expression (Schaller, 2010). In astrocytes, CNTF expression is repressed by FAK and can be increased by pharmacological FAK inhibition (Jia et al., 2018; Keasey et al., 2013). We have shown that inducible conditional knockout of FAK in astrocytes

* Corresponding author. Department of Biomedical Sciences, East Tennessee State University, Johnson City, PO Box 70582, TN, 37614, USA.
E-mail address: jiac01@etsu.edu (C. Jia).
[1] These authors contribute equally.

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increases brain CNTF but not the related cytokines leukemia inhibitory factor (LIF) and IL-6 (Jia et al., 2018, 2019b), indicating the specificity of astrocyte FAK in regulating CNTF. Our recent studies revealed a sex-specific role of CNTF in the medial amygdala (MeA) in stress responses (Jia et al., 2019a, 2022a). MeA CNTF promotes passive coping in female mice while having no effect in males. Further, chronic stress enhances passive coping in female mice via increasing MeA CNTF.

Progesterone regulates a range of physiological processes through the well-characterized classical nuclear progesterone receptors (Azeez et al., 2021) and non-classic pathways, including integrin-FAK signaling (Garg et al., 2017; Karteris et al., 2006; Shynlova et al., 2013). Our previous studies show that progesterone inhibits CNTF expression in cultured astroglioma C6 cells and mouse medial amygdala and alleviates passive stress coping behavior (Jia et al., 2019a). The mechanism underpinning progesterone-mediated CNTF inhibition is not known.

Here, we first defined whether astrocyte FAK has a sex-specific role in reducing passive coping behavior, using tamoxifen-inducible astrocyte FAK knockout mice and an FAK inhibitor. Secondly, we defined the progesterone-related mechanism underlying the female-specific role of CNTF in increasing passive coping.

2. Materials and methods

2.1. Animals

A total of 402 adult female and male mice were used. C57BL/6 (6–8 weeks old, JAX Stock 000664) and GFAP-cre breeders (B6.Cg-Tg(GFAP-cre/ERT2)505Fmv/J, JAX Stock 012849) were from the Jackson Laboratory. FAK-flox breeders (B6; 129X1-Ptk2tm1Lfr/Mmucd, RRID: MMRRC_009967-UCD) were from the MMRRC at the University of California at Davis. The Ai6 mice (B6.Cg- Gt(ROSA)26Sor $^{tm6(CAG-ZsGreen 1)Hze}$, JAX Stock 007906) were provided by Dr. Diego Rodriguez-Gil's laboratory at ETSU and GFAP-cre ZsGreen ($GFAP^{cre-ZsGreen}$) reporter mice were produced as described previously (Jia et al., 2019b). GFAP-cre FAK-flox mice ($GFAP^{cre}$-$FAK^{fl/fl}$) were bred with FAK-flox ($FAK^{fl/fl}$) to produce littermates used in experiments. Both $GFAP^{cre}$-$FAK^{fl/fl}$ and $FAK^{fl/fl}$ mice at 6–8 weeks old were treated with tamoxifen (100 mg/kg, i.p., T5648, Sigma-Aldrich) for 5 days and experiments, including forced swim test, chronic stress or tissue collection, started 2 weeks later. All mice were housed with food and water available ad libitum, and maintained on a 12 h light:12 h dark cycle. All animal procedures are consistent with the NIH Guide on Care and Use of Animals and approved by the East Tennessee State University Institutional Animal Care and Use Committee.

2.2. Chronic unpredictable stress (CUS), FAK inhibitor treatment, and behavioral tests

As described previously (Jia et al., 2022a), six environmental and social stressors, without food/water deprivation and nociceptive events, were used to induce CUS in $FAK^{fl/fl}$ and $GFAP^{cre}$-$FAK^{fl/fl}$ mice. These involved 1 h on an orbital shaker, 12 h damp bedding, 1 h immobilization, 12 h tilted cage, 1 h exposure to overcrowding, and 24 h light on. Combinations of two different stressors were applied each day over a period of 7 days (Supplemental materials). The same cycle was repeated for 4 weeks. Control mice were handled in the morning when the CUS was performed. Naïve C57BL/6 mice received three daily injections of saline or FAK inhibitor 14 (FAK14, i.p., 3, 10 or 30 mg/kg in saline, Tocris Cat# 3414). The forced swim and open field tests were performed 4 and 24 h, respectively, after the termination of CUS or the last FAK14 treatment. Both tests were conducted from 10 a.m. to 12 p.m. during the light phase, as we did previously (Jia et al., 2019a, 2022a). The forced swim test included a single 6 min trial with the last 4 min used for data analysis. The duration of immobility, defined as the cessation of all movements except those necessary to stay floating, was recorded in seconds using AnyMaze behavioral scanning software (Stoelting Co., Wood Dale, IL). The open field test measured locomotor function, serving as a control for possible motor deficits that might confound immobility behavior. The distance traveled and time spent in the center area by each mouse in a 10 min session was recorded in meter (m) by AnyMaze. For analyses of mRNA and protein, the brain was collected 4 h after termination of CUS or the last FAK14 treatment in mice that did not undergo behavioral tests.

2.3. Ovariectomy (OVX), progesterone treatment and castration

Sham, OVX or castration was performed as described previously (Jia et al., 2019a). Two weeks after surgery, vehicle (sunflower seed oil, S5007, Sigma-Aldrich) or progesterone (s.c., 10 mg/kg, Tocris Cat# 2835) was injected into sham or OVX mice for 4 days. Progesterone reportedly reduces passive coping behavior in OVX mice at a range of 0.5–40 mg/kg (Bernardi et al., 1989; Frye, 2011a, 2011b; Hansson et al., 1990; Saavedra et al., 2006). We selected a dose of 10 mg/kg that was used in multiple studies (Bernardi et al., 1989; Frye, 2011a, 2011b). This dose also produces similar plasma and brain levels of progesterone that are typically observed in mice during the estrus phase (Frye et al., 2006; Frye and Walf, 2008). Mice were subjected to the forced swim and open field tests at 4 and 24 h, respectively, after the last progesterone. For protein analysis, the brain was collected 4 h after the last progesterone in mice without behavioral tests. Castrated males were subjected to behavioral tests two weeks after surgery.

2.4. Tissue collection

Mice were anesthetized with 4% isoflurane for 30 s, followed by rapid decapitation. Trunk blood and brain were collected as we described (Jia et al., 2022a). The MeA, amygdala and hippocampus were micropunched from 700 μm thick coronal brain cryostat sections from Bregma −1.2 to −1.9 (Jia et al., 2019a, 2022a). All samples were stored at −80 °C for mRNA and protein analysis. For immunostaining, $GFAP^{Cre}$ and $GFAP^{Cre-ZsGreen}$ reporter mice were perfused with 4% paraformaldehyde, brains were cryoprotected in 30% sucrose overnight, and 20 μm thick coronal sections through the amygdala and hippocampus were cut on a cryostat.

2.5. RT-qPCR, Western blotting, immunostaining and ELISA

These analyses were performed similar to our previous work (Jia et al., 2019a, 2022a). Mouse CNTF (Mm00446373_m1), LIF (Mm00434762_g1), IL-6 (Mm00446190_m1) and GAPDH (Mm99999915_g1) primers were from ThermoFisher Scientific. Antibodies were against CNTF (MAB338, EMD Millipore, RRID: AB_2083064), phospho-FAK-Tyr397 (pFAK, #3283, Cell Signaling Technology, RRID: AB_2173659), FAK (#3285, Cell Signaling Technology, RRID: AB_2269034), β-actin (# 4967, Cell Signaling Technology, RRID: AB_330288), GFAP (MAB3402, EMD Millipore, RRID: AB_94844) and ZsGreen 1 (#632474, Clontech Laboratories, RRID: AB_2491179). Plasma levels of corticosterone were measured by ELISA (ADI-900-097, Enzo Life Sciences).

2.6. Statistical analyses

Two-tailed student t tests were used to analyze data with two groups. A one-way or two-way ANOVA was applied when there were three or more groups to test one factor or two factors, such as genotypes and treatments. The CUS effects were analyzed by repeated measure two-way ANOVA. The Bonferroni test was used for *post hoc* multiple comparisons. Statistical significance was determined by $p < 0.05$ (GraphPad Prism 7.0). Data are presented as mean ± SEM.

3. Results

3.1. Astrocyte FAK deletion or systemic treatment with FAK inhibitor increases passive coping to acute stress in females

Tamoxifen-induced cre-mediated recombination in astrocytes and subsequent deletion of FAK in the adult brain were validated in our previous studies using GFAP$^{cre-ZsGreen}$ reporter mice and PCR products (Jia et al., 2018, 2019b). We also confirmed that GFAP^{cre}-FAK$^{fl/fl}$ mice had 40% reduction of FAK protein in the frontal cortex compared to FAK$^{fl/fl}$ controls 2 weeks after the last tamoxifen treatment (Supplemental Fig. 1). Compared to the control female FAK$^{fl/fl}$ mice, GFAP^{cre}-FAK$^{fl/fl}$ females had 66% greater immobility time during a 6 min of unescapable swim stress test (Fig. 1A, $t_{(29)} = 2.193$, $p = 0.036$) while having no significant effect in males ($t_{(29)} = 0.348$, $p = 0.731$). This suggests a female-specific effect of astrocyte FAK in mitigating passive coping to acute stress. This sex-specific effect is not attributed to motor deficits since spontaneous locomotor activity tested in the open field were not different in the two genotypes in either male or female mice (Fig. 1B). Knockout of FAK in astrocytes did not affect approach/-avoidance behavior measured by the time spent in the center area of the open field (Fig. 1B). To confirm that the female-specific effect on passive stress coping is caused by tamoxifen-induced knockout, we also applied the forced swim test to female and male FAK$^{fl/fl}$ and GFAP^{cre}-FAK$^{fl/fl}$ mice without tamoxifen injections. No genotype differences were found in both sexes (Fig. 1C). Mice without tamoxifen treatment also traveled similar distance and spent comparable times in the center area of the open field (Fig. 1D). Behavioral anhedonia, anxiety-like behavior, and sensorimotor gating function tested by sucrose preference, elevated T-maze, and pre-pulse inhibition were also not different between tamoxifen-treated GFAP^{cre}-FAK$^{fl/fl}$ and FAK$^{fl/fl}$ mice (Supplemental Fig. 2). To avoid the swim stress effect, we tested these behaviors in a separate set of mice who had not undergone the forced swim test.

C57BL/6 mice received three daily systemic injections with a FAK inhibitor (FAK14), followed by forced swim and open field tests at 4 and 24 h, respectively. In females, one-way ANOVA showed a significant main effect of treatment ($F_{(3, 31)} = 6.138$, $p = 0.002$). Post hoc comparisons revealed that FAK14 at 10 and 30 mg/kg increased the immobility time 2 and 2.6 fold, respectively (Fig. 2A). In male mice, treatment with FAK14 at 10 mg/kg did not affect immobility (Fig. 2A, $t_{(17)} = 0.463$, $p = 0.325$). Higher doses were not expected to be efficacious either since full knockout has no effect. Thus, pharmacological FAK inhibition has a female-specific effect in promoting passive stress coping, possibly via inhibiting FAK in astrocytes. This sex-specific effect was not due to motor dysfunction tested in an open field test (Fig. 2B).

3.2. Astrocyte FAK knockout or systemic FAK inhibitor treatment upregulates CNTF expression in the MeA of both sexes

Next, we investigated the potential CNTF-related mechanism underlying the female-specific role of astrocyte FAK in promoting passive stress coping, focusing on the MeA (Jia et al., 2022a). CNTF mRNA and protein levels in the MeA of astrocyte FAK knockout mice were increased 2–3 fold in both sexes (Fig. 3A, female, $t_{(10)} = 3.574$, $p = 0.005$, male, $t_{(11)} = 3.668$, $p = 0.004$; Fig. 3B, mix of females and males, $t_{(16)} = 3.404$, $p = 0.004$). The CNTF-related cytokines IL-6 and LIF were comparable between FAK$^{fl/fl}$ and GFAP^{Cre}-FAK$^{fl/fl}$ mice. This is consistent with our previous finding of a very specific role of FAK in regulating CNTF (Jia et al., 2018; Keasey et al., 2013). Cre activity in MeA astrocytes was confirmed by colocalizing ZsGreen with GFAP immunostaining in GFAP$^{Cre-ZsGreen}$ reporter mice (Fig. 3C). Astrocyte FAK knockout did not affect CNTF expression in the hippocampus (Supplemental Fig. 3). Systemic FAK14 treatment at 10 mg/kg also enhanced CNTF expression in the MeA without a sex difference (Fig. 3D, mRNA, $t_{(16)} = 2.838$, $p =$

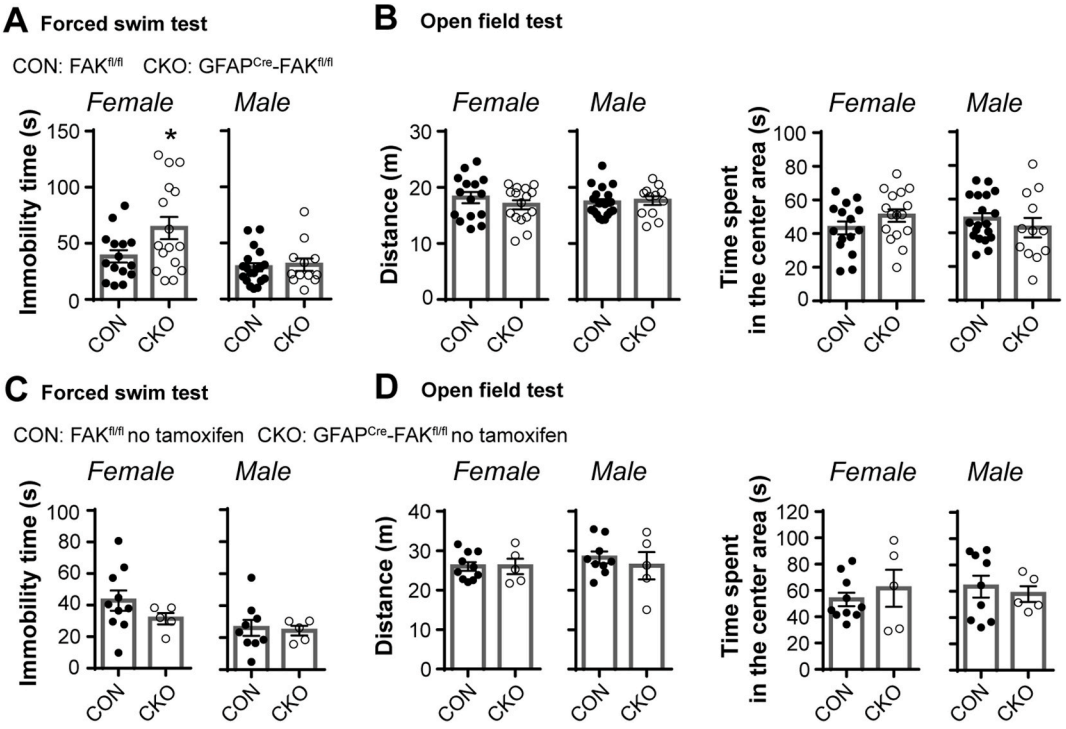

Fig. 1. Knockout of FAK in astrocytes increases immobility during acute inescapable swimming stress in female, but not male, mice. A) The FAK gene was deleted specifically in astrocytes using a tamoxifen-inducible cre-lox system. Conditional knockout (CKO, GFAP^{Cre}-FAK$^{fl/fl}$) caused an increase in passive coping compared to control littermates (CON, FAK$^{fl/fl}$) as indicated by immobility time over the last 4 min of a 6 min forced swim test only in females. **B**) FAK knockout did not affect locomotor activity and approach/avoidance behavior tested by travel distance and time spent in the center area, respectively, in an open field test in either sex. N = 15,16 females and 19,12 males. As a control, CON and CKO mice without tamoxifen treatment displayed similar behavior in the forced swim **C**) and open field test **D**) in both sexes. N = 10,5 females and 9,5 males. *p < 0.05 (Two-tailed *t*-test).

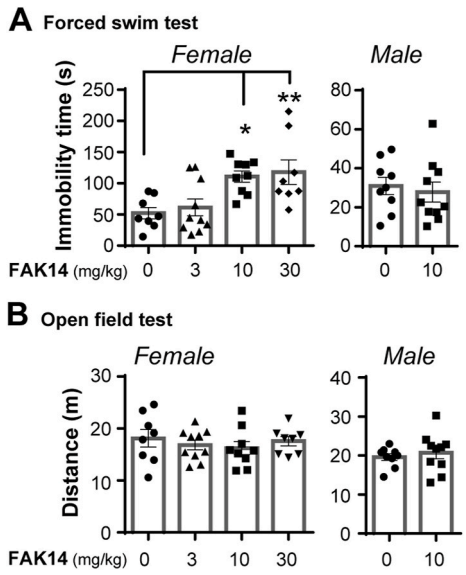

Fig. 2. Systemic treatment with an FAK inhibitor increases immobility during acute stress only in female mice. FAK inhibitor 14 (FAK14) was injected i.p. three days, followed by forced swim and open field tests at 4 and 24 h, respectively, after the last injection. **A)** In females, FAK14 at 10 or 30 mg/kg increased immobility time in the forced swim test. FAK14 at 10 mg/kg did not alter immobility time in males. **B)** FAK14 did not affect locomotor activity tested in the open field. N = 8,10,9,8 females and 9,10 males, *p < 0.05, **p < 0.01 (Females, one-way ANOVA followed by Bonferroni multiple comparisons, Males, two-tailed *t*-test).

0.012; Fig. 3E, protein, $t_{(16)} = 3.239$, $p = 0.005$, mix of females and males).

3.3. Astrocyte FAK is required for chronic stress-induced passive coping in females and chronic stress inhibits FAK and upregulates CNTF in female MeA

The immobility time in the forced swim test was measured in GFAP^{cre}-FAK$^{fl/fl}$ and FAK$^{fl/fl}$ mice before and after 4 weeks of CUS. In females (Fig. 4A), a repeated measure two-way ANOVA showed significant main effects of CUS ($F_{(1, 15)} = 21.07$, $p < 0.001$) and interaction between CUS and genotypes ($F_{(1, 15)} = 9.547$, $p=0.008$). Post hoc comparisons demonstrated that CUS increased the immobility time in female FAK$^{fl/fl}$ mice 4.5 fold, confirming the CUS effect on promoting passive coping behavior. Strikingly, CUS did not increase immobility time in GFAP^{cre}-FAK$^{fl/fl}$ females above the already high pre-CUS baseline, indicating a ceiling effect of lacking astrocyte FAK. Together, this suggests that CUS increases passive coping in females by inhibiting astrocyte FAK. In support, 4 weeks of CUS in female C57BL/6 mice reduced activated/phosphorylated FAK (pFAK) in the MeA by 90% (Fig. 4B, $t_{(10)} = 2.350$, $p = 0.041$). Consistent with our previous study (Jia et al., 2022a), CUS increased CNTF protein in female MeA (Fig. 4B, $t_{(10)} = 2.793$, $p = 0.019$). In male mice (Fig. 4C), repeated measure two-way ANOVA showed a main effect of CUS ($F_{(1, 14)} = 15.81$, $p = 0.001$). Post hoc comparisons demonstrated that CUS increased immobility time without a genotype difference, indicating that FAK in male astrocytes does not affect passive coping, which is consistent with the other data (Fig. 1A). CUS did not alter pFAK and CNTF expression in the male MeA (Fig. 4D). CUS did not alter locomotor function tested in an open field in either sex or genotype (Fig. 4A–C). Plasma corticosterone levels following CUS were comparable in both genotypes in each sex (Supplemental Fig. 4). This suggests that the lack of CUS effects in female astrocyte FAK knockout mice is not due to motor deficits or neuroendocrine changes.

3.4. Progesterone alleviates passive coping in control females and activates FAK

To determine whether progesterone contributes to the female-specific role of astrocyte FAK in inhibiting passive coping, female GFAP^{cre}-FAK$^{fl/fl}$ and FAK$^{fl/fl}$ mice were injected with progesterone for 4 days starting 14 days after OVX. A two-way ANOVA revealed significant main effects of treatments ($F_{(2, 40)} = 10.38$, $p=0.0002$) and genotypes ($F_{(1, 40)} = 5.98$, $p = 0.019$) as well as a significant interaction effect between genotypes and treatments ($F_{(2, 40)} = 27.69$, $p < 0.0001$). Post hoc comparisons showed that OVX increased immobility time 2.7 fold in FAK$^{fl/fl}$ control mice, and progesterone reversed it to the sham level (Fig. 5A). Importantly, OVX had no effect on the immobility time in GFAP^{cre}-FAK$^{fl/fl}$ females, and progesterone robustly increased, not decreased, it. This suggests that OVX-induced and progesterone-alleviated passive coping acts through astrocyte FAK. Indeed, OVX decreased pFAK and increased CNTF in the amygdala of C57BL/6 mice, which were reversed by progesterone (Fig. 5B). Together, these data suggest that progesterone-activated astrocyte FAK alleviates passive coping behavior by inhibiting CNTF expression, possibly in the amygdala. In male mice, castration did not affect the immobility time in either FAK$^{fl/fl}$ and FAK$^{fl/fl}$- GFAP^{cre} mice (Supplemental Fig. 5A), consistent with the lack of effect of astrocytic FAK knockout in males (Fig. 2B). Neither OVX, nor castration, altered motor function tested in open field tests (Fig. 5A, Supplemental Fig. 5B).

4. Discussion

This study reveals a novel female-specific role of astrocyte FAK in reducing passive coping behavior in response to inescapable acute stress. Conversely, chronic stress enhances passive coping in females by inhibiting astrocyte FAK. Importantly, the female hormone progesterone has a protective effect and systemic treatment alleviates the detrimental effects on chronic stress by activating FAK in astrocytes. Astrocyte CNTF in the MeA is one possible mechanism underlying this female-specific effect as we suggested before (Jia et al., 2022a). This is supported by the finding here that knockout of astrocyte FAK or chronic stress upregulates MeA CNTF expression in females, whereas progesterone inhibits CNTF. Thus, we have identified astrocyte FAK as a potential target for developing treatments for stress-related mental disorders in women, including depression and post-traumatic stress disorder.

4.1. Sex-dimorphic role of astrocyte FAK in acute stress coping

The switching from active (e.g., swimming) to passive (immobility, behavioral despair) coping to stress is mainly controlled by a pathway consisting of the medial prefrontal cortex, bed nucleus of the stria terminalis and periaqueductal grey (Molendijk and de Kloet, 2019; Molendijk and de Kloet, 2021). For example, chronic stress promotes passive coping behavior by attenuating excitatory output from the medial prefrontal cortex, thus releasing inhibition of the periaqueductal grey from the bed nucleus of the stria terminalis (McKlveen et al., 2016). This pathway is modulated by other brain areas, including the amygdala which plays an essential role in stress coping behavior by sending strong and direct projections to the bed nucleus of the stria terminalis (Deussing et al., 2010; Jia et al., 2019a; Jia et al., 2022a; Molendijk and de Kloet, 2021; Radley and Sawchenko, 2011). The local neurochemical mechanisms involved in amygdala-mediated regulation of passive coping are reported by multiple studies. Activation of mitogen-activated protein kinase in the amygdala increases immobility time in the forced swim test (Huang and Lin, 2006). Modulation of angiotensin, catecholamine or estrogen signaling in the MeA also affects immobility duration (Estrada et al., 2018; Kawashima et al., 1987; Marchi-Coelho et al., 2021; Moreno-Santos et al., 2021). Our previous study demonstrates that neutralizing CNTF in the MeA reduces immobility behavior in female mice only (Jia et al., 2022a). The current study suggests that the

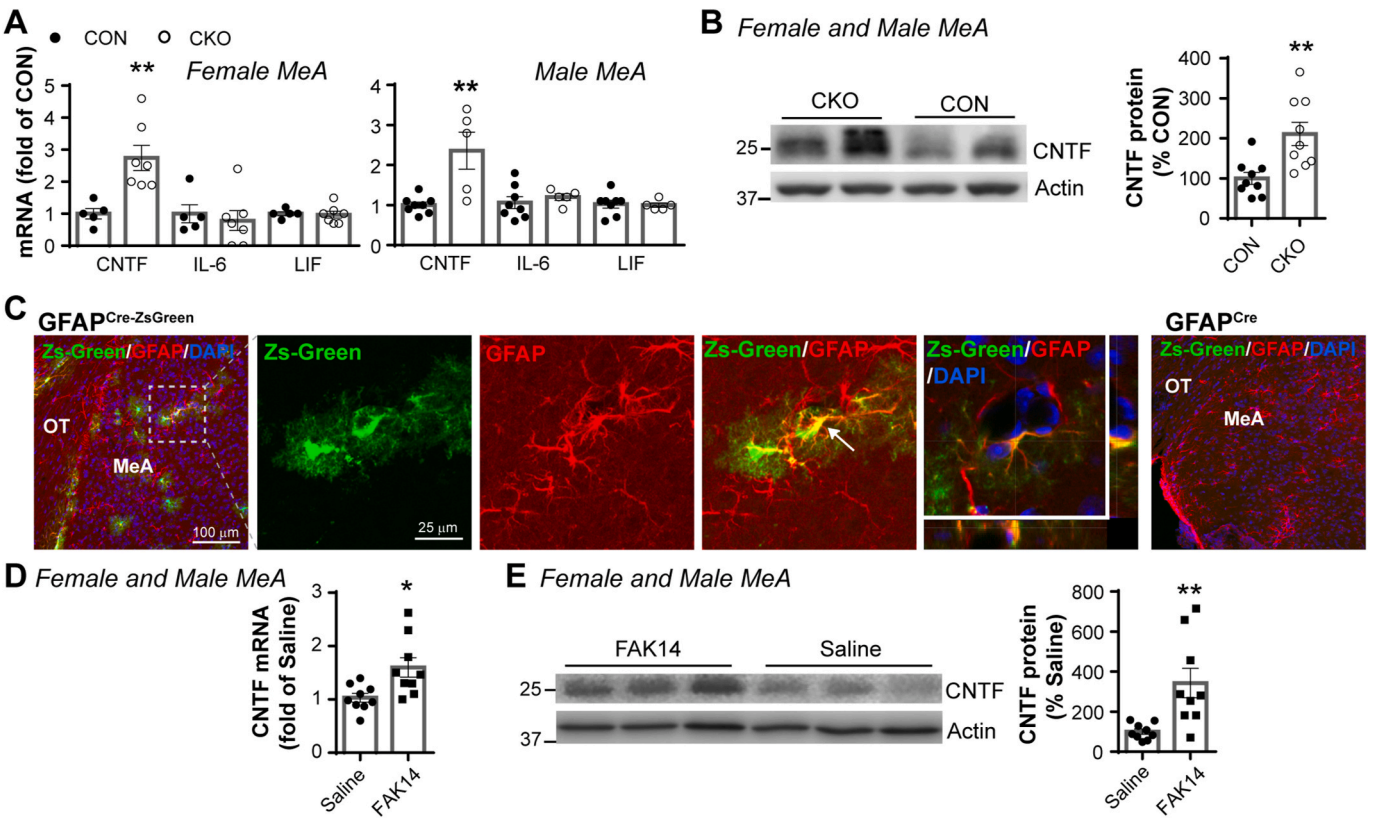

Fig. 3. Astrocyte FAK knockout or FAK inhibitor upregulates CNTF expression in the MeA of both sexes. A) $GFAP^{Cre}$-$FAK^{fl/fl}$ (CKO) female and male mice had increased CNTF, but not IL-6 and LIF, mRNA expression in the MeA. N = 5,7 females and 8,5 males. **B)** Western blots confirmed that CNTF protein was also increased, as shown by densitometry of the CNTF-immunoreactive bands. Actin served as loading control. N = 9 mice/group (mix of male and female). **p < 0.01 (Two-tailed *t*-test). **C)** Confirmation of cre recombinase-mediated ZsGreen protein expression in MeA astrocytes of $GFAP^{Cre-ZsGreen}$ reporter mice. ZsGreen immunostaining (green) is colocalized with the astrocyte marker GFAP (red). The area in the dashed square is shown at higher magnification in the next four panels. The arrow indicates the colocalization, as confirmed by X and Y projections of a single confocal image (panel 5). There was no ZsGreen positive staining in control $GFAP^{Cre}$ mice. Nuclei were counterstained with DAPI. Scale bars are as indicated. OT-optical tract. MeA-medial amygdala. **D)** Three days of FAK14 treatment (10 mg/kg, i.p.) upregulated CNTF mRNA and **E)** protein in the MeA of C57BL/6 mice (without sex difference, not shown). N = 4 females and 5 males in each group. *P < 0.05, **p < 0.01 (Two-tailed *t*-test). The representative full-length of blot stripes in B and E are shown in Supplemental Fig. 7. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

female-specific effect of astrocyte FAK on immobility might be related to increased CNTF in the MeA. Studies in human stress disorders and animal stress models reveal astrocyte dysfunction across multiple brain areas, including amygdala, such as astrocyte hypertrophy and remodeling, and reductions of connexin 30 and 43 (Murphy-Royal et al., 2019). A single bout of swim stress increases astrocyte ramification, prolongs spontaneous calcium events in fine astrocyte processes, downregulates astrocyte-specific gap junction connexin 30, and reduces astrocyte functional coupling in the prefrontal cortex (Murphy-Royal et al., 2020). These rapid structural and functional changes disrupt astrocyte metabolic networks, limit neuronal access to an astrocyte energy reservoir and impair synaptic plasticity (Murphy-Royal et al., 2020), which may result in passive coping behavior. This would be consistent with our findings that FAK inhibition greatly reduces mitochondrial bioenergetic function in cultured cells (Visavadiya et al., 2016).

4.2. Astrocyte FAK regulates stress coping by inhibiting CNTF expression

This study builds on our discovery of a FAK signaling pathway that inhibits CNTF, but not the related LIF and IL-6, expression in astrocytes (Jia et al., 2018; Keasey et al., 2013). Approximately 2–3% of human population is deficient for CNTF due to a frameshift mutation in exon 2 (Takahashi et al., 1994; Thome et al., 1997) but whether this correlates to psychiatric disorders remains ambiguous. Early studies reported that the CNTF mutation was more frequent in populations of psychiatric disorder patients than in healthy controls (Tanaka et al., 1998; Thome et al., 1996a, 1996b, 1996c). Others failed to confirm this (Gelernter et al., 1997; Grunblatt et al., 2006; Nishiyama et al., 2006; Nothen et al., 1996; Sakai et al., 1997). Given our finding that CNTF promotes passive coping behavior in females but may reduce it in males (Jia et al., 2019a), it is possible that combining data from men and women (Li et al., 1996; Nothen et al., 1996) hides a dimorphic response of CNTF deficiency. We also found that the CNTF effect in female mice is mediated by the MeA, but not the basolateral or central amygdala (Jia et al., 2022a). Our current data (Supplemental Fig. 2A) suggest that the role of astrocyte FAK is limited to only some brain regions, e.g., not the hippocampus. Our current knockout and inhibitor treatment confirm that CNTF is increased in the MeA of females in concert with increasing passive coping behavior. Together, these data suggest that chronic stress promotes passive coping in female mice via FAK inhibition-induced astrocyte CNTF, possibly in the MeA. The lack of CNTF's effect in the male MeA (Jia et al., 2022a) is consistent with the lack of effect of astrocyte FAK knockout or inhibitor treatment in the current males. We did not detect changes in amygdala IL-6 or LIF after deleting astrocyte FAK, consistent with our findings under uninjured conditions in the striatum (Jia et al., 2019b). In contrast, stroke-induced induction of detrimental IL-6 is much reduced in the injured brain tissue of astrocyte FAK knockout female, but not male, mice (Jia et al., 2022b). This suggests that the role of FAK in regulating various cytokines is dependent on the

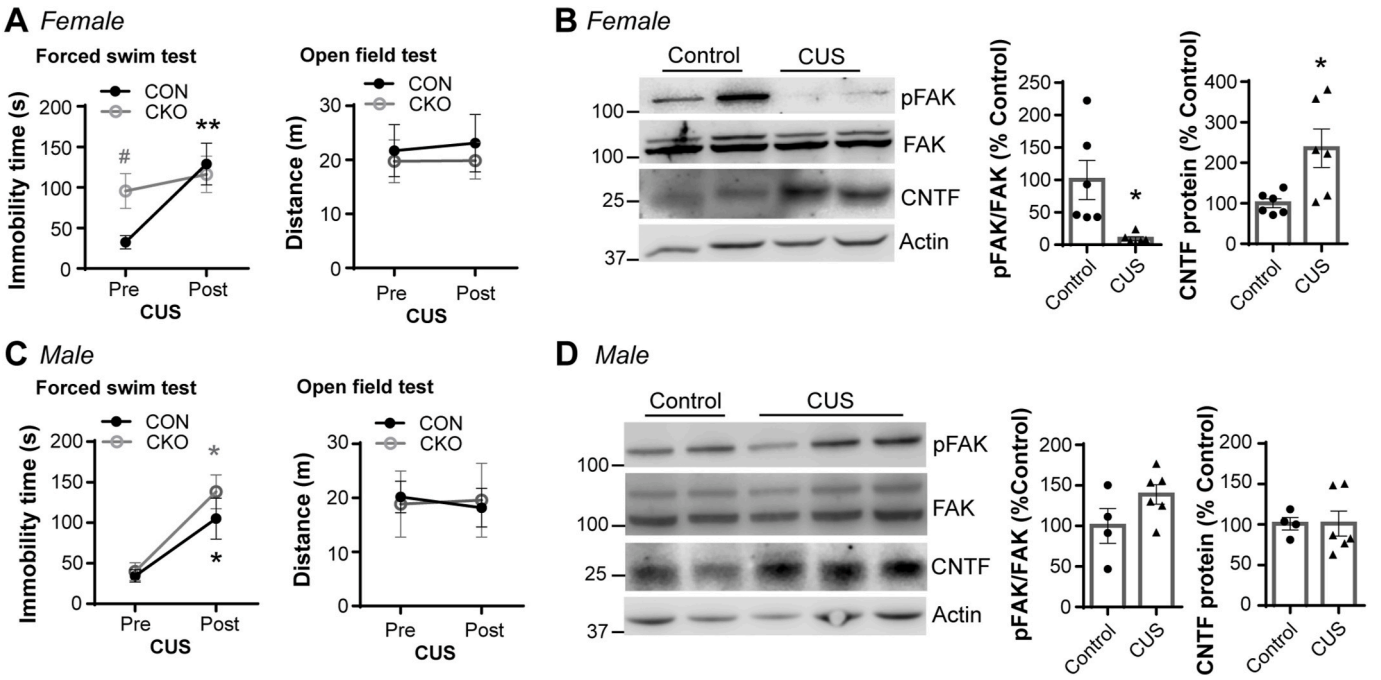

Fig. 4. Chronic stress promotes passive coping behavior in females, possibly via astrocyte FAK inhibition-induced MeA CNTF. Mice were subjected to 4 weeks of CUS. The forced swim and open field tests were applied before (pre-) and after (post-) CUS. **A**) CUS increased immobility time in the forced swim test in female control $FAK^{fl/fl}$ (CON), as expected. $GFAP^{Cre}$-$FAK^{fl/fl}$ (CKO) mice already showed an increase before CUS, and no significant further increase was seen after CUS. CUS did not alter the locomotor function tested in the open field. N = 8,9 mice. **B)** CUS markedly reduced FAK activation, as shown by phosphorylated FAK (pFAK), and increased CNTF protein in the MeA of wildtype female C57BL/6 mice, and densitometry of immunoreactive bands of the Western blots. Control mice were only handled during the same 4 weeks. N = 6,6 mice. *$p < 0.05$ (Two-tailed *t*-test). **C)** CUS increased immobility time in the forced swim test in male CON and CKO mice without altering locomotor function. N = 11,5 mice. **D)** CUS did not affect pFAK and CNTF protein in the MeA of male C57BL/6 mice. N = 4,6 mice. In A,C, *$p < 0.05$, **$p < 0.01$ vs. pre-CUS in each genotype. #$p < 0.05$ vs. CON in pre-CUS groups (Two-way repeated measure ANOVA followed by Bonferroni multiple comparisons). The representative full-length of blot stripes in B and D are shown in Supplemental Fig. 8.

nature of diseases or disorders. Chronic stress and stress-related disorders have been associated with increased brain inflammation, including IL-6 expression (Fuchs et al., 2013; McKim et al., 2016). Thus, further investigation into the underlying mechanism(s) would be needed to develop FAK-activating treatments for women with different disease pathologies.

4.3. Role of progesterone in regulating astrocyte FAK in chronic stress responses

Our data suggest that FAK mediates passive coping behavior in response to CUS in a female-specific manner. CUS inhibited FAK and promoted CNTF expression in the female MeA, suggesting that this is a key regulating signaling pathway. Moreover, CUS did not affect passive coping which was already high after knockout of astrocyte FAK. It will be important to determine the mechanism by which CUS inhibits astrocyte FAK. CUS reduces the expression of connexin 43 in the rodent prefrontal cortex (Giaume et al., 2010; Miguel-Hidalgo et al., 2018; Sun et al., 2012). In glioma stem cells and some cancer models, connexin 43 directly interacts with FAK (Tien et al., 2021; Zhou et al., 2020) and regulates FAK activity by recruiting Src, Src inhibitors, as well as phosphatase and tensin homolog (Jaraiz-Rodriguez et al., 2017; Qin et al., 2015). Stress also induces extensive extracellular matrix (ECM) remodeling and modulates integrin expression and function, possibly by stimulating ECM-degrading enzyme, leading to reduced integrin binding (Jean et al., 2011). Thus, CUS-induced FAK inhibition in females could be mediated by such mechanisms.

Our data show that progesterone activates FAK and reduces CNTF in the amygdala and alleviates OVX-induced passive stress coping in female control but not astrocyte FAK knockout mice, indicating progesterone acts through the astrocyte FAK-CNTF pathway. In apparent conflict, progesterone treatment after OVX increased immobility in astrocyte FAK knockout mice (Fig. 5A). Neither OVX nor progesterone altered CNTF levels in the amygdala (Supplemental Fig. 6), indicating that progesterone-induced immobility behavior in astrocyte FAK knockout mice is independent of CNTF. We do not know the mechanism (s) underlying this effect. It is possible that this mechanism also exists in WT mice but that it is overruled by the FAK-CNTF pathway. Other mechanisms that can increase immobility time in the forced swim include progesterone acting through modulating serotonin and 5-HT2 receptors (Kaur and Kulkarni, 2002).

Progesterone regulates a range of physiological processes through the well-characterized classical nuclear progesterone receptors (Azeez et al., 2021). Progesterone also acts through non-classic pathways, including membrane-bound progesterone receptors and integrin-FAK signaling (Garg et al., 2017; Karteris et al., 2006; Shynlova et al., 2013). Progesterone promotes neuronal spine formation via membrane progesterone receptor-mediated activation of FAK (Sanchez et al., 2013). Progesterone is metabolized to dihydroprogesterone (DHP) by 5α-reductase and DHP is further reduced to allopregnanolone. Progesterone-reduced passive coping occurs through membrane-bound progesterone receptors and allopregnanolone action at GABA receptors (Beckley and Finn, 2007; Beckley et al., 2011; Frye et al., 2004). Here, we show that progesterone also does so by activating FAK in astrocytes.

Reductions of ovarian hormones, including progesterone, are thought to contribute to the greater susceptibility of women to stress. The incidence of stress-related symptoms is higher when ovarian hormone levels are low, including premenstrual, postpartum, and perimenopausal periods (Hiroi and Neumaier, 2011). Thus, normal or high ovarian hormone levels seem to be protective. Both estrogen and progesterone mitigate passive stress coping behavior in rodent models

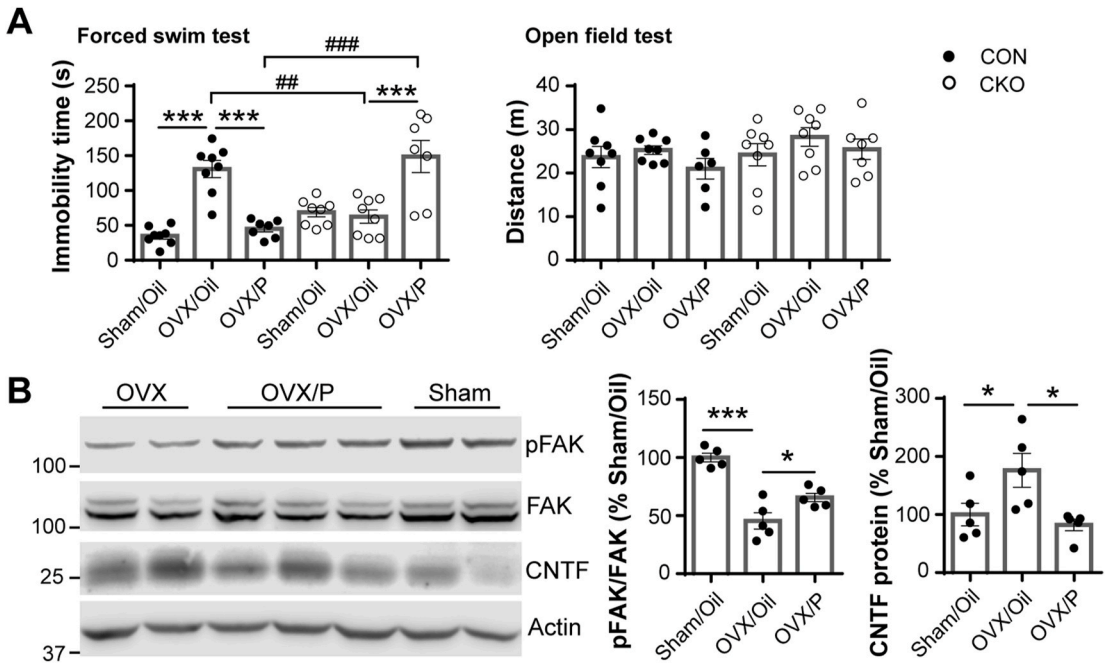

Fig. 5. Progesterone alleviates passive coping by activating astrocyte FAK. Four days of sunflower seed oil (vehicle) or progesterone (10 mg/kg s.c.) treatment was applied to sham-operated or ovariectomized (OVX) mice 2 weeks after surgery. **A)** OVX caused an increase in immobility times in the forced swim test in control (CON, $FAK^{fl/fl}$), which was prevented by progesterone. Knockout of astrocyte FAK (CKO, $GFAP^{Cre}$-$FAK^{fl/fl}$) prevented the OVX-induced passive coping behavior. Progesterone did not further decrease it as in CON mice, but, instead, caused an unexplained increase. OVX or progesterone did not alter locomotor function tested in an open field. N = 8,8,7,8,8,7 mice. ##p < 0.01, ###p < 0.001, ***p < 0.001 (Two-way ANOVA followed by Bonferroni multiple comparisons). **B**) OVX reduced FAK activation (pFAK) and increased CNTF protein in the amygdala compared to sham C57BL/6 mice as shown in Western blots and their densitometry values. These changes were reversed by 4 days of progesterone treatment. N = 5,5,5 mice. *p < 0.05, ***p < 0.001 (One -way ANOVA followed by Bonferroni multiple comparisons). The representative full-length of blot stripes in B are shown in Supplemental Fig. 9.

by modulating neurotransmission and increasing hippocampal BDNF (Douma et al., 2005; Frye, 2011a). Our data identify another mechanism of astrocyte FAK. Progesterone receptors are enriched in the amygdala (Brinton et al., 2008). Astrocytes express classic progesterone receptors and progesterone receptor membrane component 1 (PGRMC1) (Bali et al., 2013). PGRMC1 has target sequences for binding SH2- and SH3-domain proteins and kinases, implicating a possible role as an adaptor protein to regulate intracellular signal transduction (Cahill, 2007). Whether PGRMC1 activates FAK signaling in astrocytes needs further study. Progesterone in the circulation and brain is sensitive to stress. Chronic stress affects plasma progesterone differently depending on the stress paradigms, estrous cycle, pregnant stages and assessment methods (Anderson et al., 1996; Goncalves et al., 2022; MacNiven et al., 1992; Serra et al., 2000; Wilsterman et al., 2018; Zhu et al., 2021). Astrocytes are the primary cell synthesizing progesterone in the brain (Sinchak et al., 2003) and various stress paradigms decrease progesterone and its metabolisms in the brain (Barbaccia et al., 2001; Serra et al., 2000; Weisz et al., 1982). It remains to be determined whether CUS inhibits FAK by reducing brain progesterone signaling.

Progesterone inhibits passive coping behavior and suppresses CNTF expression in the amygdala (Jia et al., 2019a). Our current data suggest that progesterone does so through FAK activation. Progesterone seems to have a specific role as it, but not estrogen, inhibits CNTF expression in astroglioma C6 cells (Jia et al., 2019a). Progesterone did affect CNTF in the hippocampus (Jia et al., 2019a) but we cannot exclude its effects and roles in other brain areas. Chronic stress does not alter CNTF in several other stress-related brain areas of female mice, including the paraventricular nucleus or bed nucleus of the stria terminalis (Jia et al., 2022a). In contrast, deletion of microRNA-155 reduces passive coping in both sexes but increases hippocampal CNTF in female mice only (Fonken et al., 2016), confirming that hippocampal CNTF is not involved in the sex-specific effect of our data. Astrocyte FAK in the amygdala seems to affect a limited number of behaviors as anhedonia measured by sucrose preference, anxiety-like behavior measured by elevated T maze, or sensorimotor gating function measured by pre-pulse inhibition, were not affected by the knockout. This is also consistent with the lack of effects on those behaviors by CNTF knockout (Jia et al., 2019a, 2022a).

5. Conclusion

Together, this study reveals a novel astrocyte FAK-mediated, female-specific, mechanism in stress responses and points to opportunities for developing treatments for stress-related disorders in women.

CRediT authorship contribution statement

Cuihong Jia: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **W. Drew Gill:** Data curation, Formal analysis, Investigation, Methodology. **Chiharu Lovins:** Data curation, Formal analysis, Methodology. **Russell W. Brown:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Theo Hagg:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no competing financial interests or potential conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2024.100621.

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