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Sex differences in social interaction behaviors in rats are mediated by extracellular signal-regulated kinase 2 expression in the medial prefrontal cortex

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Abstract

Considerable sex differences occur in the incidence and prevalence of anxiety disorders where women are more anxious than men, particularly in situations where social interaction is required. In preclinical studies, the social interaction test represents a valid animal model to study sex differences in social anxiety. Indeed, female rats engage less in conspecific interactions than their male counterparts, which are behaviors indicative of higher social anxiety in female rats. In this work, we implicated extracellular signal regulated kinase 2 (ERK2) in the medial prefrontal cortex (mPFC) in mediating social interaction. Indeed, female rats' had lower ERK2 expression compared to male rats, and overexpression of ERK2 in the mPFC increases their social interaction to the level seen in their male counterparts. These data indicate that the sexually dimorphic expression of ERK2 mediates social anxiety-like behaviors.

Keywords

anxiety; ERK2; social interaction; sex differences; prefrontal cortex

Introduction

Anxiety disorders are some of the most common mental disorders affecting nearly 1 in 5 adults in the United States (Kessler et al., 2005), and they afflict women significantly more than men (Angst and Dobler-Mikola, 1985, Regier et al., 1990, Bruce et al., 2005). In addition to higher overall lifetime prevalence rates for general anxiety disorders, social anxiety disorder is also more prevalent in women than men (Kessler et al., 1994). Although sex differences in the incidence and prevalence of anxiety disorders are well established, many animal studies exploring the neurobiological mechanisms underlying anxiety-like behaviors have been carried out solely in males. Undoubtedly, more studies delving into the mechanisms mediating sex differences in anxiety responses are necessary.

The social interaction test measures anxiety-like behaviors in the context of environment-dependent social behavior and has been shown to have a high degree of ethological validity.

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Sex differences have been previously reported in the social interaction test by our group and others (Johnston and File, 1991, Stack et al., 2010, Slamberova et al., 2011), such that male rats spend more time engaging in social interaction with an unfamiliar conspecific than their female counterparts. Social anxiety is inversely proportional to the amount of time participating in social interaction behaviors (File and Hyde, 1978).

In our previous work, we have shown that the sexually dimorphic expression of the immediate early gene *zif268* in the medial prefrontal cortex (mPFC) mediates sex-specific anxiety-like behavior in the social interaction test (Stack et al., 2010). To further investigate the molecular mechanisms in the mPFC underlying sex differences in social anxiety behaviors, we hypothesized that there might be sexually dimorphic expression of extracellular signal-regulated kinase 2 (ERK2), a direct activator of *zif268* (Khachigian and Collins, 1997, Silverman and Collins, 1999, Osawa et al., 2004).

The mitogen activated protein kinase (MAPK) pathway constitutes a complex intracellular signaling network implicated in a variety of cellular and emotional responses. Interestingly, sex specific changes in MAPK-ERK2 activation have been recently reported (Cuffe et al., 2011). Within the mPFC, activation of the MAPK-ERK2 pathway is required for the consolidation and recall of recent memories (Sweatt, 2001) and occurs following administration of antipsychotic and antidepressant drugs (Alboni et al., 2010, Pereira et al., 2011). Furthermore, several studies report decreased phosphorylated ERK2 in the prefrontal cortex following stress exposure (Meller et al., 2003, Shen et al., 2004, Qi et al., 2006), suggesting that MAPK-ERK2 signaling in the prefrontal cortex is critical in the stress response and may be a principle component implicated in the molecular pathophysiology of mood disorders.

Herein, we have explored the implications of MAPK-ERK2 signaling in the mPFC in underlying sex differences in social anxiety-like behaviors in adult rats. We identified sexually dimorphic ERK2 expression in the mPFC as a key molecular factor in mediating sex-specific social interaction behaviors. The characterization of a novel role for MAPK-ERK2 signaling within the mPFC highlights the significance of this cascade and provides new information on the molecular mechanisms underlying social anxiety.

Materials and Methods

Experiment 1: Expression of components of the MAPK pathway in the mPFC of male and cycling female rats

Adult male (n=6) and cycling female rats in their diestrus (n=6) or proestrus (n=6) stage were sacrificed under basal non-stressful conditions. The mPFC (dorsal and ventral) was dissected out and ERK1, ERK2, and MEK2 (ERK kinase) mRNA and total ERK1/2 and phosphorylated ERK1/2 protein expression levels were determined.

Experiment 2: Overexpression of ERK2 in the mPFC of male and female rats using herpes simplex viral vector constructs

The herpes simplex virus (HSV) expressing ERK2 coupled to green fluorescent protein (wtERK2-GFP; n=8) or expressing GFP alone (n=8), was injected directly into the prelimbic mPFC of male and female rats. Three days following the initial injection, GFP expression was visualized using fluorescence microscopy. Separately, protein expression of total ERK1/2 and phosphorylated ERK1/2 was examined via Western blotting.

Experiment 3: Overexpression of ERK2 in the mPFC increases social interaction behavior in female rats

Male (n=12) and female (n=14) resident rats were injected with wtERK2-GFP or GFP alone and their social interaction behaviors were tested 3 days later in a large open field arena with an unfamiliar intruder rat of the same age, weight and sex. The intruder rats were gonadectomized/ovariectomized to eliminate any possible hormonal influence on the behavior of the resident rat. The resident rats were sacrificed immediately after the social interaction session for injection site placement verification.

Animals

Adult male (weighing 250–270g) and female (weighing 200–225g) Sprague-Dawley rats, were purchased from Charles River (Wilmington, MA, USA). All rats were pair-housed in 43×21.5×25.5cm plastic cages and kept on a 12h: 12h light: dark cycle (lights on at 0700 hours). Food and water was available *ad libitum* except during testing. All behavioral experiments were conducted during the first 4 h of the light phase of the light/dark cycle and all animal protocols were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Florida State University.

Surgery—Rats were anesthetized with a ketamine (70 mg/kg)/xylazine (10 mg/kg) mixture (i.p.). Bupivacaine (0.25% solution; 0.4mL/kg) was applied topically as analgesic and the non-steroidal anti-inflammatory drug meloxicam (1.0 mg/mL) was injected subcutaneously.

Gonadectomy/Ovariectomy—A 1–2 cm ventral midline incision was made in the scrotum of adult male rats to expose the tunica. The tunica was pierced and both testes were extracted to expose the underlying blood vessels, which were ligated with silk suture. The testes were excised and all vessels and ducts were placed back into the tunica prior to suturing. A slightly larger 2–3 cm ventral midline incision was made in the lower abdominal region of adult female rats to expose the uterus. Visible blood vessels were ligated, the ovaries removed, and the muscle layers and skin were sutured.

Vaginal lavage—Vaginal smears were performed daily on female rats to determine the stage of estrus cycle. As previously described (Stack et al., 2010), diestrus was defined by the presence of small numbers of leukocytes. Proestrus was defined by the presence of clumps of large round nucleated epithelial cells.

Viral vector infusions and transgene detection—HSV vectors containing GFP or wtERK2-GFP, were a generous gift from Dr. Eric Nestler (Mount Sinai School of Medicine, NY). These vectors have been previously described, and validated *in vivo* and *in vitro* (Krishnan et al., 2007, Russo et al., 2007, Carrier and Kabbaj, 2012). The average titer of the recombinant virus stocks was 4.0×10^7 infectious units/mL. For stereotaxic delivery of the viral constructs, rats were bilaterally microinjected (1.0 μ l per side over 10 min) into the mPFC (anteroposterior, +2.7 mm; lateral, $\pm$ 1.0 mm; dorsoventral, –3.0 mm from bregma (Paxinos G, 1998). Our group and others have previously shown that maximal transgene expression for these vectors occurs 3–4 days after injection (Carlezon et al., 1998, Carrier and Kabbaj, 2012). To verify peak GFP expression in the mPFC, rats were injected with HSV-GFP (n=3) and were transcardially perfused 3 days later with phosphate buffered saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted, postfixed at 4°C in paraformaldehyde overnight, sectioned at 30 μ m on a vibratome (Leica VT1000S, Buffalo Grove, IL), and GFP expression observed under a confocal microscope (Leica TCS SP2, Buffalo Grove, IL). GFP expression was visible in the cingulate, prelimbic,

and infralimbic regions of the mPFC and all rats had correct placements between +2.2 and +3.7 from bregma (see figure 3).

Social Interaction Test—Testing was performed as previously described (Stack et al., 2010). Briefly, the resident rat was habituated to an open field arena (1m × 1m) for 10 min, and an unfamiliar conspecific gonadectomized/ovariectomized rat was then placed in the arena with the resident for an additional 10 min. The locomotor activity and time in the center during the first 10 min, and the time spent by the resident rat engaging in active social interaction during the second 10 min was measured using EthoVision XT version 6 (Noldus Information Technology, Leesburg, VA).

Semi-Quantitative Real-Time PCR—Total RNA was extracted from the mPFC and complimentary DNA synthesis was carried out as previously described (Hollis et al., 2010, Carrier and Kabbaj, 2012). Nicotinamide adenine dinucleotide dehydrogenase (NADH) was used as the reference gene for normalization of all target genes. The forward and reverse primer sequences were as follows:

ERK1: 5'-GTTCTGACATCCCTGGAGGA-3'; 5'-CCAGGGCAGAGAACTACTGG-3'

ERK2: 5'-GACAAGGGCTCAGAGGACTG-3'; 5'-ACGGCTCAAAGGAGTCAAGA-3'

MEK2: 5'-GCATTCCCGAGGACATCTTA-3'; 5'-TTGGAGGGCTTCACATCTCT-3'

NADH: 5'-CTATTAATCCCCGCCTGACC-3'; 5'-GGAGCTCGATTGTTTCTGC-3'

The normalized data is expressed as percent of control, with male rats set at 1.

Western Blot—Total proteins were extracted from the dorsal and ventral mPFC, or from the injection site to confirm HSV viral construct efficacy. Protein samples were processed as described previously (Carrier and Kabbaj, 2012). Briefly, immunoblots were incubated overnight (4°C) with ERK1/2 (Cell Signaling Technology; 1: 1000) and GAPDH (Cell Signaling Technology; 1: 1000), or phospho-ERK1/2 (Cell Signaling Technology, 1: 1000) and ERK1/2 (1: 1000) antibodies, washed and incubated 1 h with goat anti-rabbit IR Dye 680LT (Li-COR Biosciences; 1: 10000) or goat anti-mouse IR Dye 800CW (Li-COR; 1: 10000) fluorescent secondary antibodies, and visualized using an Odyssey infrared imaging system (Li-COR Biosciences). Quantification was done using NIH ImageJ (<http://rsbweb.nih.gov/ij>). Normalized data are expressed as percent of control, with control males set at 1.

Statistical analysis—Results were analyzed using one-way or two-way analysis of variance (ANOVA) followed by post-hoc Fisher tests where appropriate. P values < 0.05 were considered statistically significant.

Results

Expression of components of the MAPK pathway in the mPFC of male and cycling female rats

Male, diestrus female, and proestrus female rats did not differ in MEK2 (Figure 1A; $F_{(2,12)} = 0.705$; $p > 0.05$) or ERK1 ($F_{(2,12)} = 0.861$; $p > 0.05$) mRNA expression in the mPFC. ERK2 expression was different in the mPFC such that both diestrus female and proestrus female

rats had decreased mRNA compared to male rats ($F_{(2,9)} = 5.726$; $p < 0.05$). ERK2 mRNA expression was similar between diestrus and proestrus females.

Western blot analysis also revealed sexually dimorphic ERK2 expression (Figure 1B; $F_{(2,16)} = 4.724$; $p < 0.05$) confirming decreased ERK2 expression within the mPFC in diestrus and proestrus females compared to male rats. ERK2 protein expression was similar between diestrus and proestrus females. ERK1 ($F_{(2,8)} = 1.380$; $p > 0.05$), phosphorylated ERK1 ($F_{(2,8)} = 2.444$; $p > 0.05$), and phosphorylated ERK2 ($F_{(2,8)} = 1.507$; $p > 0.05$) protein expression were similar in the mPFC of diestrus female, proestrus female, and male rats.

Overexpression of ERK2 in the mPFC of male and female rats using herpes simplex viral vector constructs

The GFP construct exhibited maximal expression in the mPFC 3 days after injection (Figure 2A) throughout the mPFC from +2.2 through +3.7 from bregma (Figure 3A–B). The wtERK2-GFP construct increased ERK2 expression in the mPFC compared to the GFP control (Figure 2B; $F_{(1,12)} = 13.727$; $p < 0.05$) but did not affect ERK1 ($F_{(1,12)} = 0.247$; $p > 0.05$), phosphorylated ERK1 ($F_{(1,12)} = 4.448$; $p > 0.05$), or phosphorylated ERK2 ($F_{(1,12)} = 2.217$; $p > 0.05$) expression. The wtERK2-GFP construct increased ERK2 expression in both male (Figure 2C; $F_{(1,7)} = 5.586$; $p < 0.05$) and female ($F_{(1,5)} = 10.962$; $p < 0.05$) rats.

Overexpression of ERK2 in the mPFC increased social interaction behavior in female rats

Locomotor activity, measured as the number of grid crosses in the open field was not affected by sex ($F_{(1,24)} = 3.180$; $p > 0.05$) or by overexpression of ERK2 in the mPFC (Figure 4A; $F_{(1,24)} = 0.713$; $p > 0.05$). Similarly, ERK2 overexpression (Figure 4B; $F_{(1,24)} = 0.260$; $p > 0.05$) and sex ($F_{(1,24)} = 0.678$; $p > 0.05$) had no effect on the time spent in the center of the open field. Social interaction behavior was affected by sex (Figure 4C; $F_{(1,24)} = 0.0034$; $p < 0.05$) and there was a sex by treatment interaction ($F_{(1,24)} = 4.534$; $p > 0.05$). The wtERK2 construct did not affect social interaction behaviors in male rats compared to the GFP control ($F_{(1,9)} = 0.200$; $p > 0.05$), but increased social interaction behaviors in female rats to the level seen in male rats ($F_{(1,12)} = 5.914$; $p < 0.05$).

Discussion

In the social interaction test, social anxiety-like behaviors are negatively correlated with the time spent engaging in active social interaction. This phenomenon has been empirically described and validated with anxiolytic and anxiogenic drugs (File and Hyde, 1978, Johnston and File, 1989). Although the nature of the interactions are not entirely understood, prominent sex differences in social interaction, where male rats spend more time engaging in social interaction than their female counterparts in the resident-intruder paradigm are well established (Johnston and File, 1991, Stack et al., 2010). The present study provides evidence that sexually dimorphic ERK2 expression (decreased in female compared to male rats) in the mPFC mediates sex differences in social interaction. Increasing ERK2 expression in the female mPFC resulted in increased social interaction behaviors that were comparable to that seen in male rats. This effect was specific to social anxiety-like behavior as the same treatments did not affect anxiety-like behaviors measured in the open field test. Indeed, ERK2 expression in the mPFC may be a key component in mediating sex differences in social anxiety.

The prefrontal cortex is an important regulator of social behavior and cognition. Specifically, abnormal functioning within the prefrontal cortex results in social inappropriateness, lack of judgment, inappropriate affect, and impairments in social decision making (Bechara et al., 1999, Barrash et al., 2000). In our previous study, we established

that sexually dimorphic zif268 expression in the mPFC mediates sex differences in social interaction behaviors. In fact, reducing zif268 expression in the mPFC of male rats decreased social interaction behavior and increased social anxiety (Stack et al., 2010). Since zif268 is a downstream target of ERK2, the present study suggests that decreased zif268 expression and social interaction in females might be linked to decreased ERK2 expression in the mPFC (Khachigian and Collins, 1997). Taken together, these studies implicate a key role for decreased expression of ERK2 and zif268 in the mPFC of female rats in mediating decreased social anxiety-like behaviors. Interestingly, manipulation of either ERK2 or zif268 expression did not affect general anxiety-like behaviors as measured by the open field test, suggesting that the observed effects are specific to social anxiety-like behaviors and may not extrapolate to other forms of anxiety.

The MAPK-ERK pathway is a major convergence point in many signaling pathways and although there are no documented studies of a specific association with social anxiety disorders, there are implications for the MAPK pathway in affective disorders. For instance, preventing ERK1/2 activation through inhibition of MEK1/2 induced depressive-like behaviors in male mice (Duman et al., 2007). Furthermore, stress-mediated disruption of ERK1/2 signaling induced depressive-like symptoms and this effect was reversed by fluoxetine treatment (Qi et al., 2008). Phosphorylation of ERK1/2 often leads to activation of multiple transcription factors including c-fos, c-jun, Arc, and zif268, resulting in an intricate signaling network (Xia et al., 1996, English and Sweatt, 1997, Guzowski et al., 2000) capable of mediating complex behaviors. Herein, we investigated the possibility that the activation of ERK1/2 via phosphorylation in the mPFC might also be sexually dimorphic. While our findings demonstrate sex differences only in basal ERK2 expression, future investigations are warranted to examine the possibility of sexually dimorphic targets downstream of ERK2.

Neurobiological sex differences in signaling cascades, such as the MAPK pathway and its downstream targets, are likely set during development when the brain is exposed to the organizational effects of hormones. Thus, it is likely that inherent sex-specific differences in molecular signal transduction in the mPFC mediate sexually dimorphic social anxiety behavior in the adult. It is not clear why social interaction behaviors in male rats did not increase following overexpression of ERK2 in the mPFC. Similarly, in our previous study (Stack et al., 2010), reducing zif268 expression in the mPFC of female rats did not decrease their social interaction behaviors. These observations are likely due to ceiling and flooring effects in the expression levels of zif268 and ERK2 in the mPFC. Furthermore, there may also be sex differences in critical downstream targets of zif268 and ERK2 that are implicated in sex-specific social interaction behaviors. Future investigations are needed to test these hypotheses.

Sex differences in MAPK gene expression are often attributable to estrogen. For example, estrogen has been shown to induce CREB-mediated gene activation by activating the MAPK pathway in a sex specific manner (Barabas et al., 2006). However, we observed decreased ERK2 expression in the mPFC of female rats in both diestrus (low estrogen) and proestrus (high estrogen) stages of the estrus cycle. Thus, reduced ERK2 expression in female rats and the lack of regulation during the estrus cycle suggests a potential role for testosterone in maintaining high levels of ERK2 in the mPFC of male rats. In fact, testosterone activates the MAPK-ERK pathway (Migliaccio et al., 2000, Cheng et al., 2007) and recent evidence from our group showed testosterone-dependent regulation of hippocampal ERK2 expression in mediating depressive-like behaviors in male rats (Carrier and Kabbaj, 2012). The contribution of gonadal hormones in ERK expression in the mPFC and in social anxiety requires further investigation.

We have demonstrated that sexually dimorphic expression patterns of ERK2 in the mPFC can mediate sex-specific differences in social anxiety-like behaviors. These findings highlight how a key molecular player in a complex signaling cascade can contribute to a behavioral outcome and raise many more important questions concerning the neurobiological mechanisms underlying sex differences in behavior and psychopathologies.

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- Female rats have higher social anxiety than their male counterparts
- Female rats have lower expression of ERK2 in the mPFC
- ERK2 expression in the mPFC mediates sex differences in social anxiety

Figure 1.

Expression of MAPK components in the mPFC in male and cycling female rats. Compared to males, female rats in diestrus and proestrus exhibit decreased ERK2 (A) mRNA and (B) protein expression in the mPFC. * $p < 0.05$ compared to male.

Figure 2.

Overexpression of ERK2 in the mPFC. Injection of HSV constructs into the mPFC elicited (A) extensive GFP expression and (B) increased ERK2 expression, but not ERK1 or phosphorylated ERK1/2 protein expression by the wt-ERK2 in (C) both male and female rats on day 3. * $p < 0.05$ compared to GFP control.

Figure 3.

HSV vector injection placement. (A) Photomicrograph showing representative placement of the lesion created during HSV injection into the mPFC and illustrating the subsequent location of GFP expression three days after HSV-GFP injection. (B) Representative images adapted from *The Rat Brain in Stereotaxic Coordinates* (Paxinos G, 1998) illustrating the total area (+2.2 to +3.7 from bregma) affected by stereotaxic injection.

Figure 4.

Anxiety-like behaviors in male and female rats three days after injection of HSV constructs into the mPFC. Overexpression of ERK2 had no effect on (A) locomotor activity and (B) time spent in the center of the open field but (C) increased social interaction behaviors in female rats to the level of social interaction seen in male rats. * $p < 0.05$ compared to GFP