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Overexpression of angiotensin converting enzyme 2 reduces anxiety-like behavior in female mice

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Abstract

Accumulating evidence has revealed an intricate role for the renin-angiotensin system (RAS) in the progression or alleviation of stress-related disorders. Along these lines, the 'pro-stress' actions of angiotensin-II (Ang-II) are largely thought to be mediated by the angiotensin type-1a receptor (AT1aR). On the other hand, a counter regulatory limb of the RAS that depends on the conversion of Ang-II to angiotensin-(1-7) by angiotensin-converting enzyme 2 (ACE2) has been postulated to exert stress-dampening actions. We have previously found that augmenting ACE2 activity is potentially anxiolytic and blunts stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis in male mice. Whether increasing ACE2 activity also relieves stress and anxiety in females has not yet been determined. Consequently, this series of experiments tests the hypothesis that augmenting ACE2 expression is anxiolytic and dampens the activity of the HPA axis in female mice. Using the Cre-LoxP system, we generated female mice that were homo-, heterozygous or wild-type for a mutated allele resulting in ubiquitous overexpression of ACE2. Next, we used qPCR to determine that levels of ACE2 mRNA isolated from central and peripheral tissues was dependent on genotype. That is, mice homo- and heterozygous for the ACE2 overexpression had significantly greater levels of ACE2 mRNA relative to littermate matched wild-type controls. Interestingly, anxiety-like behavior as determined by the elevated plus maze, light-dark box and novelty-induced hypophagia tests was also affected by genotype. Specifically, ACE2 overexpression significantly decreased anxiety-like behavior in paradigms dependent on approach-avoidance conflict and novelty; however, locomotor activity was similar amongst the genotypes. Final experiments measured plasma corticosterone to evaluate whether increasing ACE2 alters basal and/or stress-induced HPA axis activity. In contrast to what was previously

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found in males, increasing ACE2 expression had no effect on plasma corticosterone under basal conditions or subsequent to an acute restraint challenge. Collectively, these results suggest that increasing ACE2 expression potentially elicits anxiolysis in female mice without altering HPA axis activity.

1. Introduction

The prevalence of stress-related disease is increasing in the U.S. and it is estimated that ~ 19% of adults suffer from anxiety disorders and nearly 30% will be afflicted at some point in their lifetime (Harvard-Medical-School 2007a, Harvard-Medical-School 2007b). Females have an increased susceptibility to stress-related diseases, like anxiety and depression (Kessler, McGonagle et al. 1993, Kessler, Berglund et al. 2003); however, the majority of the pre-clinical research aimed at identifying causes and treatments for affective disorders is conducted in male animal models (Zucker and Beery 2010, Beery and Zucker 2011). Consequently, pre-clinical studies using female non-human subjects are needed to evaluate whether potential interventions for alleviating affective disorders are efficacious in both sexes.

Emerging evidence suggests that the renin-angiotensin-system (RAS) may be a viable therapeutic target for relieving stress and anxiety (Saavedra, Sanchez-Lemus et al. 2011). The effector peptide of the RAS, angiotensin II (Ang-II), is synthesized through proteolytic cleavage events whereby renin converts angiotensinogen into angiotensin I, which is cleaved by angiotensin converting enzyme into Ang-II, which in turn, activates the angiotensin type 1_a receptor (AT1_aR). Prior studies have found that stressful stimuli induce Ang-II binding to AT1_aR(s) but antagonizing this interaction attenuates indices of stress and anxiety-like behavior in male rodents (Castren and Saavedra 1988, Saavedra, Ando et al. 2005, Krause, de Kloet et al. 2011, Marvar, Goodman et al. 2014). More recently, the therapeutic utility of angiotensin converting enzyme 2 (ACE2) and the counter-regulatory limb of the RAS has been pursued as an experimental therapeutic for stress-related disease. Angiotensin converting enzyme 2 limits the production of Ang-II and AT1_aR activation by cleaving an amino acid from Ang-II to form Ang (1–7) which binds to the Mas receptor (MasR). Genetic or pharmacological upregulation of ACE2 activity decreases endocrine, cardiovascular and behavioral responses to stress in male rodents (Martins Lima, Xavier et al. 2013, Wang, de Kloet et al. 2016, Wang, de Kloet et al. 2018); however, whether similar effects occur in females has not been investigated.

Here, we use female mice genetically-engineered to ubiquitously overexpress ACE2 to investigate whether increased ACE2 activity influences behavioral and endocrine indices of stress responding. Initial experiments used real-time qPCR to measure ACE2 mRNA expression in tissues obtained from wild-type controls and ACE2 overexpressing mice. Next, mice were tested in experiments assessing anxiety-like behavior and hypothalamic-pituitary-adrenal (HPA) axis activity. Collectively, the results suggest that increasing ACE2 activity is potentially anxiolytic in female mice.

2. Methods

2.1 Animals

All mice were female and 8–12 weeks old at the beginning of the studies. Mice were individually-housed with *ad libitum* access to rodent chow and water unless otherwise noted. The University of Florida Institutional Animal Care and Use Committee approved all procedures and protocols.

Ubiquitous overexpression of ACE2 was accomplished using the Cre-LoxP system as previously described (Wang, de Kloet et al. 2016). Briefly, the mouse ACE2 gene was *FLAG*-tagged and expressed under the regulation of the endogenous ROSA26 promoter on one allele, creating *ROSA26^{ACE2/+}* mice. The *FLAG*-tagged ACE2 containing cassette was preceded by a *floxed NEO-STOP* cassette which was placed after the *ROSA26* promoter to generate *ROSA26^{Floxed Neo-STOP, ACE2/+}* mice. Mice with Cre recombinase expression directed to the β -actin gene were crossed with the *ROSA26^{Floxed Neo-STOP, ACE2/+}* mice to remove the *floxed Neo-STOP* cassette and create *ROSA26^{ACE2/+; β -actin-Cre/+}* mice. β -actin Cre was then removed through several series of breeding steps to produce *ROSA26^{ACE2/+}* mice which were subsequently maintained on a 129/B6 mixed background. To generate cohorts of mice of all three genotypes, heterozygous *ROSA26^{ACE2/+}* mice were bred to each other to achieve homozygous (HOM; 25%) and heterozygous (HET; 50%) ACE2 knock-in mice (ACE2KI) as well as wild type (WT; 25%) littermate controls. Female mice that are HET or HOM for ACE2 overexpression are viable and fertile.

2.2 Tissue dissection and quantitative real-time polymerase chain reaction (qPCR)

Brains were extracted and flash frozen in dry ice-cooled 2-methylbutane and pituitaries and adrenals were collected and frozen on dry ice. Tissue was then stored in the -80°C until processing. To remove the amygdala and hypothalamus, brains were placed in a brain block cooled in a cryostat chamber (-17°C ; Leica CM3050 S). Razor blades were passed through the brain block to isolate thick coronal sections (4 mm) containing the amygdala and hypothalamus. Sections were then placed on the cryostat stage and the amygdala was isolated using a 1.5 mm tissue punch (Stoelting, Wood Dale, IL). The hypothalamus was isolated by removing tissue that was dorsal to the optic tracts and lateral to the 3rd ventricle. Collection and dissection of tissue was performed under RNase-free conditions. RNA was extracted from brain dissections and from whole pituitary and adrenal glands using Trizol (Invitrogen). RNA extraction, cDNA synthesis, and real-time PCR were conducted as previously described (de Kloet, Pati et al. 2013). Probes for real-time PCR were purchased from Applied Biosystems (ThermoFisher) and specific genes of interest included ACE2 (Mm0159003) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Mm99999915), which was used as the house-keeping gene. Relative expression was quantified using the $2^{-\text{Ct}}$ method.

2.3 Evaluating anxiety-like behavior

Elevated plus maze (EPM)—The EPM was used as a proxy for generalized anxiety-like behavior. The apparatus is elevated 41 cm off of the floor and consists of two open arms (31 cm x 6 cm) opposite each other and two opposite closed arms (31 cm x 6 cm). Both ACE2KI

and control mice were tested in the EPM during the light phase between 8:00AM and 12:00PM. Each mouse was placed in the center of the maze facing an open arm and had five minutes to explore. Sessions were recorded using TopScan software (CleverSys, Reston, VA).

Light-dark box (LDB)—The LDB was used as another proxy of generalized anxiety-like behavior. It consists of an arena (40 cm x 40 cm x 35 cm) where half the arena (40 cm x 20 cm) is a clear “light side” with an open top and the other half (40 cm x 20 cm) is a “dark side” with opaque walls and a closed top. The two sides are joined by an opening where mice can freely-move between them. ACE2KI mice and WT littermates were tested in the LDB between 8:00AM and 12:00PM during the light phase. Each mouse was placed in the dark side and allowed five minutes to explore the maze. Each session was recorded using TopScan software (CleverSys, Reston, VA).

Novelty-induced hypophagia (NIH)—Novelty-induced hypophagia uses the latency to consume a familiar palatable food in a novel environment as a proxy for anxiety-like behavior in mice (Merali, Levac et al. 2003). ACE2KI and control mice were habituated to graham crackers (0.2 g) in their home cage at the same time each day for a minimum of five days and latencies to consume the graham cracker were recorded with a stop watch. Subsequent to habituation, mice were introduced to a novel environment (a standard mouse cage with opaque metallic-colored walls) and given access to the graham cracker. Mice had 5 minutes access to the graham cracker in the novel environment and latencies to consume were again recorded.

2.4 Measurement of plasma corticosterone

We measured basal plasma corticosterone levels during the circadian nadir (between 0800 h and 0900 h) and circadian peak (between 1700 h and 1800 h) of corticosterone secretion, and again before, during and after a 30 minute restraint challenge in ACE2KI and control mice. For the restraint challenge, the test was initiated between 0800 h and 0900 h. Mice were restrained in clear plastic ventilated tubes and blood samples (40 µl) were collected from the tail vein < 3 minutes after the onset of restraint. Thirty-minutes later, another blood sample was collected and mice were released from the restraint tubes and placed into home cages. Additional blood samples were collected after 60 and 120 minutes of recovery. Blood samples were centrifuged at 3500 rpm for 15 minutes at 4 °C to isolate plasma. Plasma samples were stored at –80 °C. Plasma corticosterone concentrations were measured using an I¹²⁵ radioimmunoassay kit (MP Biomedicals, Orangeburg, NY) as previously described (Krause, Melhorn et al. 2008, Krause, de Kloet et al. 2011).

2.5 Statistical analyses

All data were analyzed and graphed using Prism 8 (Graphpad Software, La Jolla, Ca) and presented as mean and SEM. Gene expression, EPM and LDB data were analyzed using one-way ANOVA and Tukey’s post hoc analyses. Corticosterone and NIH data were analyzed using two-way repeated measures ANOVA and Bonferroni’s post hoc analyses. In all cases, significance was set at $p < 0.05$.

3. Results

3.1 Cre-Lox recombination increases the expression of ACE2 mRNA in the brain, pituitary and adrenal

Previous studies have verified the efficacy of Cre-Lox mediated overexpression of the ACE2 allele in male ACE2KI mice relative to WT controls (Wang, de Kloet et al. 2016). In order to confirm that these alterations in ACE2 expression also occur in females, we used real-time qPCR to measure ACE2 mRNA levels in tissues obtained from ACE2KI and control mice. In the amygdala, there was an effect of genotype ($F(2, 14) = 27.74$, $p < 0.0001$) with mice HOM or HET for the ACE2 KI allele having significantly higher levels of ACE2 mRNA than wild-type littermate controls (Figure 1A). Additionally, ACE2 mRNA expression was significantly increased in HOM ACE2KI mice when compared to that of HET ACE2KI mice (Figure 1B). Similar results were observed when ACE2 mRNA was measured in the hypothalamus ($F(2, 15) = 54.93$, $p < 0.0001$; Figure 1B), pituitary ($F(2, 7) = 32.76$, $p = 0.0003$; Figure 1C) and adrenal ($F(2, 15) = 66.26$, $p < 0.0001$; Figure 1D). That is, HOM and HET ACE2KI mice both had significantly increased ACE2 mRNA relative to WT littermate controls. Furthermore, HOM mice had significantly elevated ACE2 mRNA relative to their HET counterparts. Collectively, these results indicate that Cre-Lox mediated overexpression results in augmented ACE2 gene expression in a manner that is dependent on genotype.

3.2 ACE2 overexpression attenuates anxiety-like behavior in female mice

We conducted a variety of behavioral assays to evaluate the effects that ACE2 overexpression has on anxiety-like behavior in female mice. As shown in Figure 2, ACE2 overexpression significantly affected anxiety-like behavior in the EPM. Statistical analysis revealed an effect of genotype, with HOM ACE2KI mice spending more time in the open arms (Figure 2A, $F(2, 54) = 6.35$, $p < 0.003$), and, having more open arm entries (Figure 2B, $F(2, 54) = 3.59$, $p < 0.0345$) of the EPM relative to HET ACE2KI mice and wild-type littermate controls; however, the total distance traveled in the EPM was similar amongst the groups (Figure 2C).

To determine whether the anxiolytic effects of ACE2 overexpression persisted in another test of anxiety-like behavior, mice were evaluated in the LDB. As can be seen in Figure 3, there was a significant effect of genotype on the percentage of time spent in ($F(2, 27) = 4.82$, $p = 0.02$) and on the number of entries into ($F(2, 27) = 3.73$, $p = 0.04$) the light side of the LDB. Specifically, HOM and HET ACE2KI mice spent over twice as much time in the light side and also entered the light side twice as many times as their WT littermates; however, the latencies to enter the light side were similar among the groups.

The EPM and LDB are similar tests of anxiety-like behavior in that both depend on the rodents' natural affinity for dimly-lit spaces. Consequently, we conducted the NIH test to determine whether ACE2-induced suppression of anxiety-like behavior persists in a behavioral assay that relies on novelty to elicit an approach-avoidance conflict. As depicted in Figure 4, there were no differences in the latency to consume the graham cracker in home cages subsequent to the habituation period. In contrast, latency to consume the graham

cracker in a novel environment was significantly decreased in HOM and HET ACE2KI mice relative to wild-type littermate controls (significant genotype by environment interaction; $F(2, 26) = 3.8$, $p = 0.04$). Collectively, these results suggest that increasing ACE2 expression in females may promote resiliency to stress, in part, by reducing anxiety-like behavior.

3.3 ACE2 overexpression does not affect HPA axis activity in female mice

We measured plasma levels of corticosterone in the morning, evening and subsequent to a restraint challenge in order to determine whether ACE2 overexpression alters basal or stress-induced HPA axis activity. Figure 5A depicts the basal morning and evening levels of plasma corticosterone in HOM and HET ACE2KI mice and their WT littermate controls. As can be seen, there was an effect of time ($F(1, 33) = 106.4$, $p < 0.0001$) with plasma corticosterone levels being significantly increased in the evening relative to the morning. However, no effect of genotype was detected with HOM, HET and WT mice exhibiting similar plasma corticosterone levels in the morning and evening. Figure 5B highlights plasma corticosterone before, during and after a 30-minute restraint challenge. As expected, there was again an effect of time ($F(2.33, 69.9) = 124.4$, $p < 0.0001$) with plasma corticosterone significantly increasing at 30 and 60 minutes relative to samples taken at 0 and 120 minutes. However, there was no effect of genotype with HOM, HET and WT mice demonstrating similar basal, stress-induced and recovery levels of plasma corticosterone. Taken together, these results suggest that ACE2 overexpression does not influence basal or stress-induced HPA axis activation in female mice.

4. Discussion

The goal of the present study was to determine whether increasing ACE2 expression is associated with anxiolysis and HPA axis-dampening in adult female mice. Towards this end, we used the Cre-LoxP system to generate female mice that were homo-, heterozygous or wild type for ACE2 overexpression. We then used a variety of behavioral tests to assess anxiety-like behavior and measured basal and stress-induced levels of plasma corticosterone. An effect of ACE2 overexpression was observed on indices of anxiety-like behavior in the EPM, LDB and NIH tests. In contrast, we found no effect of ACE2 overexpression on basal morning and evening levels of plasma corticosterone. Stress-induced elevations in plasma corticosterone were also unaffected. Collectively, these results suggest that ACE2 overexpression attenuates anxiety-like behavior but has no effect on HPA axis activity in adult female mice.

To the best of our knowledge, ours is the first study to examine whether increasing the activity of the counter-regulatory limb of the RAS influences anxiety-like behavior in females. Genetic or pharmacological up-regulation of ACE2 activity in the brain was previously found to decrease anxiety-like behavior in male mice (Wang, de Kloet et al. 2016, Wang, de Kloet et al. 2018). As mentioned, ACE2 cleaves Ang-II to form Ang-(1–7), which is an endogenous ligand for the MasR. Interestingly, when ACE2 overexpressing mice were centrally-administered the MasR antagonist, A-779, the anxiolysis associated with increased central ACE2 activity was abolished (Wang, de Kloet et al. 2016). These results suggested that activation of MasR(s) residing within the CNS is anxiolytic, which is consistent with

prior studies demonstrating that knockout of the MasR increases anxiety-like behavior in male mice (Walther, Balschun et al. 1998) but delivery of Ang-(1–7) into the brain blunts cardiovascular responses to stress in male rats (Kangussu, Almeida-Santos et al. 2013, Martins Lima, Xavier et al. 2013, Almeida-Santos, Kangussu et al. 2016, Kangussu, Almeida-Santos et al. 2017). The present study found that female mice overexpressing ACE2 exhibited increased approach behavior as indicated by spending more time investigating anxiogenic environments relative to controls. Along the same lines, the latency to consume palatable food in a novel environment was also decreased in female mice with ACE2 overexpression. Taken together, the results from our behavioral experiments demonstrate that ACE2 overexpression reduces anxiety-like behavior in females, and suggest that therapies targeting ACE2 activity in the brain may alleviate behavioral symptoms of affective disorders in both sexes.

In addition to altered behavior, affective disorders are also associated with endocrine abnormalities, like exaggerated HPA axis activity (Naughton, Dinan et al. 2014, Sze and Brunton 2019). Consequently, we also evaluated whether ACE2 overexpression altered HPA axis activity in females but found no effect on basal or stress-induced elevations in plasma corticosterone, results that contrast what was previously-found in males (Wang, de Kloet et al. 2018). That is, ubiquitous overexpression of ACE2 in male mice results in normal basal but blunted stress-induced elevations in plasma corticosterone (Wang, de Kloet et al. 2018). Activation of the HPA axis is initiated by increased firing of neurons in the paraventricular nucleus of the hypothalamus (PVN) that synthesize corticotropin-releasing-hormone (CRH). These neurons send axons to the median eminence and release CRH into a portal vascular system, which triggers the synthesis of proopiomelanocortin (POMC) and the secretion of adrenocorticotropin hormone (ACTH) from the pituitary, which in turn, promotes the adrenal release of corticosterone. Interestingly, male mice with ubiquitous ACE2 overexpression were found to have lower levels of POMC mRNA in the pituitary, suggesting that increasing ACE2 may suppress stress-induced activation of the HPA axis by down-regulating the POMC precursor, CRH. Consistent with this interpretation, coupling ACE2 overexpression to the CRH gene recapitulated the anxiolytic and HPA axis dampening that occurred with ubiquitous overexpression (Wang, de Kloet et al. 2018). These results, in conjunction with those from the present study, suggest that ACE2 overexpression may not similarly affect the function of CRH neurons controlling the HPA axis in females.

Relative to males, females exhibit a heightened HPA axis response to acute stressors (Sze and Brunton 2019). Specifically, following an acute restraint challenge, female rats have higher levels of circulating CORT (Babb, Masini et al. 2013, Babb, Masini et al. 2013), augmented activation of the PVN (Babb, Masini et al. 2013), and increased CRH expression (Iwasaki-Sekino, Mano-Otagiri et al. 2009) when compared to that of males. In addition, females appear to have enhanced sensitivity to CRH and ACTH at the levels of the pituitary and adrenal, respectively (Figueiredo, Ulrich-Lai et al. 2007, Goel, Workman et al. 2014). The increased PVN activation and CRH expression, in particular, suggest that acute stressors initiate HPA axis activation to a greater extent in females relative to males. Therefore, it is reasonable to hypothesize that ACE2 overexpression fails to blunt stress-induced elevations in plasma corticosterone because its inhibitory effects on CRH neurons succumbs to the augmented HPA axis activation that occurs in females. Consistent with this hypothesis,

ACE2 overexpression directed specifically to the CRH gene attenuates plasma corticosterone 30 minutes after the onset of acute stress, indicating that an ACE2-CRH interaction blunts initial activation of the HPA axis in male mice (Wang, de Kloet et al. 2018). Interestingly, the heightened HPA axis activity that occurs in females is mediated, in part, by ovarian hormones as ovariectomy lowers basal and stress-induced HPA axis activity (Seale, Wood et al. 2004). In this regard, prior studies suggest that estrogens influences ACE2 expression within systemic tissues and this may affect stress-induced HPA axis activation (Gupte, Thatcher et al. 2012, Wang, Jessup et al. 2013). More than likely, ovarian hormones and ACE2 interact in females to alter HPA axis activity and additional research is needed to address this possibility.

Stress-related diseases, like affective disorders, can affect males and females differently. In particular, the prevalence of anxiety disorders in females is nearly twice that of males (McLean, Asnaani et al. 2011, Craske, Stein et al. 2017) and symptomology, disease progression and treatment may differ depending on sex. Historically, the RAS has been targeted for alleviating cardiometabolic disease, the etiology and treatment of which, differs depending on sex (Gerdtz and Regitz-Zagrosek 2019). In this regard, women with cardiovascular disease require lower doses of ACE inhibitors or AT1_aR blockers than men to achieve optimal effects (Santema, Ouwerkerk et al. 2019). Interestingly, ACE2 is linked to the X chromosome, meaning females carry two copies of the gene and augmented ACE2 activity may contribute to cardiometabolic improvements in females. Here, we propose that the counter-regulatory limb of the RAS, especially ACE2, can also be targeted to alleviate affective disorders in females.

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Figure 1. Female ACE2KI mice exhibit robust elevations in ACE2 gene expression in selected brain areas and peripheral tissues.

Relative ACE2 mRNA levels in the (A) amygdala (AMY; $n = 5 - 8/\text{group}$), (B) hypothalamus (HYP; $n = 5 - 8/\text{group}$), (C) pituitary (PIT; $3 - 4/\text{group}$) and (D) adrenal (ADR; $n = 5 - 7/\text{group}$) of female WT, HET and HOM mice. * = significantly different from WT ($P < 0.05$). # = significantly different from HET ($p < 0.05$). Bars represent SEM.

Figure 2. ACE2 overexpression in female mice attenuates anxiety-like behavior in the Elevated-Plus Maze (EPM).

(A) Percentage of time spent in the open arms, (B) number of entries into the open arms and (C) total distance traveled during the 5 minute test. $n = 12\text{--}24/\text{group}$. * = significantly different from WT ($P < 0.05$). # = significantly different from HET ($p < 0.05$). Bars represent SEM.

Figure 3. ACE2 overexpression in female mice reduces anxiety-like behavior in the LDB. (A) Percentage of time spent, (B) number of entries and (C) latency to enter into the light side of the LDB. $n = 8-11$ /group. * = significantly different from WT ($P < 0.05$). Bars represent SEM.

Figure 4. ACE2 overexpression reduces novelty-induced hypophagia (NIH) in female mice. The latency to approach Graham Cracker crumbs in the Home cage of the mouse (left) or in a Novel environment (right). $n = 8-12/\text{group}$. * = significantly different from WT ($P < 0.05$). # = significantly different from Home for the same genotype ($P < 0.05$). Bars represent SEM.

Figure 5. ACE2 overexpression does not impact the hypothalamic-pituitary adrenal axis in female mice.

(A) Corticosterone levels assessed during the circadian nadir (AM) and circadian peak (PM) of secretion. $n = 9 - 14$ / group. * = significantly different than AM corticosterone value for the same genotype, $p < 0.05$. (B) Corticosterone response to an acute 30-minute restraint challenge. * = significantly different than basal “0” corticosterone value (significant for all three genotypes), $p < 0.05$. $n = 8 - 13$ /group. Bars represent SEM.