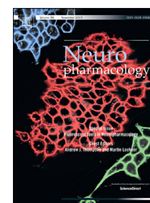
Neuropharmacology 105 (2016) 114–123

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm



Increasing brain angiotensin converting enzyme 2 activity decreases anxiety-like behavior in male mice by activating central Mas receptors

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ARTICLE INFO

Article history:
Received 23 September 2015
Received in revised form
4 December 2015
Accepted 31 December 2015
Available online 6 January 2016

Keywords:
Anxiety
ACE2
Mas receptor
Basolateral amygdala
GABA
Stress

ABSTRACT

Over-activation of the brain renin-angiotensin system (RAS) has been implicated in the etiology of anxiety disorders. Angiotensin converting enzyme 2 (ACE2) inhibits RAS activity by converting angiotensin-II, the effector peptide of RAS, to angiotensin-(1–7), which activates the Mas receptor (MasR). Whether increasing brain ACE2 activity reduces anxiety by stimulating central MasR is unknown. To test the hypothesis that increasing brain ACE2 activity reduces anxiety-like behavior via central MasR stimulation, we generated male mice overexpressing ACE2 (ACE2 KI mice) and wild type littermate controls (WT). ACE2 KI mice explored the open arms of the elevated plus maze (EPM) significantly more than WT, suggesting increasing ACE2 activity is anxiolytic. Central delivery of diminazene aceturate, an ACE2 activator, to C57BL/6 mice also reduced anxiety-like behavior in the EPM, but centrally administering ACE2 KI mice A-779, a MasR antagonist, abolished their anxiolytic phenotype, suggesting that ACE2 reduces anxiety-like behavior by activating central MasR. To identify the brain circuits mediating these effects, we measured Fos, a marker of neuronal activation, subsequent to EPM exposure and found that ACE2 KI mice had decreased Fos in the bed nucleus of stria terminalis but had increased Fos in the basolateral amygdala (BLA). Within the BLA, we determined that ~62% of GABAergic neurons contained MasR mRNA and expression of MasR mRNA was upregulated by ACE2 overexpression, suggesting that ACE2 may influence GABA neurotransmission within the BLA via MasR activation. Indeed, ACE2 overexpression was associated with increased frequency of spontaneous inhibitory postsynaptic currents (indicative of presynaptic release of GABA) onto BLA pyramidal neurons and central infusion of A-779 eliminated this effect. Collectively, these results suggest that ACE2 may reduce anxiety-like behavior by activating central MasR that facilitate GABA release onto pyramidal neurons within the BLA.

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1. Introduction

Excessive uncontrollable anxiety is the most prevalent mental illness with ≈ 18% of adults in the U.S. being afflicted with some sort of anxiety disorder (Kessler et al., 2005). Despite therapeutic advancements, it is estimated that 40% of patients with anxiety disorders are resistant to treatment (Bystritsky, 2006). While medications such as benzodiazepines are effective at relieving anxiety, they are also associated with severe side effects such as sedation, memory impairments, tolerance and dependence (reviewed by (Uzun et al., 2010)). Accordingly, it is imperative to identify new medications that effectively relieve anxiety without deleterious side effects.

Patients with anxiety have increased risk for developing cardiovascular disease (Johannessen et al., 2006; Thurston et al., 2013) and a recent epidemiological study found that symptoms of post-traumatic stress disorder (PTSD), a type of anxiety disorder, are less severe in patients taking anti-hypertensive medications that target the renin-angiotensin-system (RAS) (Khoury et al., 2012). The effector peptide of the RAS is synthesized through a series of proteolytic cleavage events whereby renin converts angiotensinogen into angiotensin-I, which is cleaved by angiotensin converting enzyme (ACE) into angiotensin-II (Ang-II), which in turn, activates

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http://dx.doi.org/10.1016/j.neuropharm.2015.12.026
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the angiotensin type 1_a receptor ($AT_{1a}R$). Several preclinical studies have found that stressful and anxiogenic stimuli promote Ang-II induced activation of $AT_{1a}Rs$ but inhibiting this receptor or the synthesis of Ang-II dampens stress responsiveness and relieves fear and anxiety-like behavior in laboratory rats and mice (Castren and Saavedra, 1988; Krause et al., 2011b; Marvar et al., 2014; Saavedra et al., 2005). Taken together, these results suggest that over-activation of the ACE/Ang-II/$AT_{1a}R$ axis contributes to the etiology of anxiety disorders and therapies that limit its activation may be anxiolytic.

The relatively recent discovery of angiotensin converting enzyme 2 (ACE2) revealed a counter-regulatory limb of the RAS that opposes many of the deleterious consequences of $AT_{1a}R$ activation (reviewed by (Xu et al., 2011)). Angiotensin converting enzyme 2 metabolizes Ang-II into angiotensin 1–7 (Ang-(1–7)) which promotes cardio-protection by activating the Mas receptor (MasR) to cause vasodilation and decreased sympathetic nervous system activity (reviewed by (Xia and Lazartigues, 2010)). Emerging evidence suggests that this counter-regulatory limb of the RAS also influences physiological and behavioral responses to psychological stress. Genetic deletion of the MasR increases anxiety-like behavior in mice (Walther et al., 1998) but central administration of Ang-(1–7) blunts cardiovascular reactivity in response to psychogenic stress (Martins Lima et al., 2013) and attenuates anxiety-like behavior in rats (Bild and Ciobica, 2013; Kangussu et al., 2013). The relatively short half-life ($\approx$10s) of Ang-(1–7) delivered *in vivo* in rats (Yamada et al., 1998) limits its therapeutic utility; however, ACE2 activity can be sustained pharmacologically in rats (Qi et al., 2013) and has the advantage of lowering Ang-II levels while also providing ligand for the MasR. Anti-hypertensive therapies have targeted the RAS for decades (Gavras et al., 1978), and consequently, it is established that interventions that suppress RAS activity are not associated with the deleterious side effects (e.g. sedation, dependence, memory impairment) that occur with some of the current anti-anxiety medications (Townsend, 2015). Therefore, ACE2 may be well-suited to serve as a therapeutic target to relieve anxiety; however, preclinical studies evaluating the effects of ACE2 on anxiety-like behavior have not been conducted.

The present study used genetic and pharmacological approaches in mice to test the hypothesis that increasing ACE2 in the brain reduces anxiety-like behavior by activating central MasRs. To identify brain circuits mediating the anxiolytic effects of ACE2, we assessed Fos induction within limbic regions subsequent to an anxiogenic stimulus. Experiments utilizing *in situ* hybridization and *in vitro* brain slice electrophysiology were conducted to reveal the neuronal phenotypes affected by MasR stimulation. Together, these studies suggest increasing brain ACE2 activity may be a novel therapeutic strategy for alleviating anxiety disorders.

2. Materials and methods

2.1. Animals

All mice were male, 8–12 weeks-old at the initiation of the studies, individually-housed and given *ad libitum* access to pelleted rodent chow and water. All procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

2.1.1. ACE2 overexpressing mice

Using *Cre-Lox* technology, *ROSA26*$^{Ace2/+}$ mice (created by Dr. Oh, University of Florida) with the *FLAG*-tagged mouse ACE2 gene expressed under the control of the endogenous *ROSA26* promoter on one allele were generated. Briefly, a gene cassette containing mouse *ACE2-Flag* proceeded by a *floxed Neo-STOP* cassette was inserted after the *ROSA26* locus to generate *ROSA26*$^{Floxed\ Neo\text{-}STOP,Ace2/+}$ mice. Subsequently, *ROSA26*$^{Floxed\ Neo\text{-}STOP,Ace2/+}$ mice were crossed with germline *Cre* deleter mice (β-actin-Cre) to remove the *floxed Neo-STOP* cassette and thus generate *ROSA26*$^{Ace2/+;\ \beta\text{-}actin\text{-}Cre/+}$ mice. β-actin-Cre was then removed through a series of breeding steps to produce *ROSA26*$^{Ace2/+}$ mice. *ROSA26*$^{Ace2/+}$ mice were then inbred to generate *ROSA26*$^{Ace2/Ace2}$ (ACE2 KI) and wild-type (WT) littermate control mice that were maintained on a 129/B6 mixed background. For all studies utilizing ACE2 KI mice, comparisons were made to their corresponding wild-type littermate controls with the exception of *in vitro* electrophysiological experiments which made comparisons between ACE2 KI mice and wild-type mice derived from similar parental lines.

2.1.2. GABA-reporter mice

Mice that have Cre-recombinase directed to GABA synthesizing cells [vGAT-Cre; The Jackson Laboratory, stock # 016962] were bred to mice with a mutation of the Gt(ROSA)26 Sor locus with a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven sequence coding for the red fluorescent protein variant, tdTomato (Jackson Laboratory Stock # 007914). Mice co-expressing vGAT-Cre and loxP-flanked STOP-tdTomato have the loxP-flanked STOP cassette deleted specifically in vGAT-producing (i.e. GABAergic) cells, resulting in tdTomato expression specifically in GABAergic cells. To confirm that tdTomato expressing cells were indeed GABAergic we performed RNAscope *in situ* hybridization (Advanced Cell Diagnostics, Hayward, CA) to visualize GAD1 mRNA, a marker of GABA synthesis, in tdTomato expressing cells (Please refer to section 2.8).

2.1.3. C57BL/6 mice

Additional studies utilizing C57BL/6 mice obtained from Harlan Laboratories were conducted to evaluate the effect of diminazene aceturate (DIZE; ACE2 activator) or A-779 (MasR antagonist) on anxiety-like behavior.

2.2. Assessment of hydromineral balance

Tail-vein blood samples (40 μl) were collected from ACE2 KI mice ($n = 12$) and WT littermates ($n = 9$). Subsequently, plasma sodium, hematocrit, and protein levels were measured as previously described (Frazier et al., 2013; Krause et al., 2011a; Smith et al., 2014).

2.3. Tissue dissection and semiquantitative real-time polymerase chain reaction (PCR)

Brains, pituitary glands, and adrenal glands were extracted from another group of mice (ACE2 KI mice $n = 9$; WT littermates $n = 11$) and flash frozen in dry ice cooled 2-methylbutane. To dissect the hypothalamus and amygdala, the brain was placed in a cooled brain block and a brain slice (4 mm) containing the hypothalamus and amygdala was isolated using razor blades. The amygdala was removed from the brain slice using a tissue punch (1.50 mm; Stoelting, Wood Dale, IL). The hypothalamus was isolated by removing tissue dorsal and lateral to the hypothalamus with a razor blade. RNA extraction, cDNA synthesis, and real-time PCR were conducted as previously described (de Kloet et al., 2013). Probes for real-time PCR were purchased from life technology (Grand Island, NY) and specific genes of interest included ACE2 (Mm01159003), $AT_{1a}R$, (Mm01166161), angiotensin type-2 receptor (AT_2R; Mm01341373), and MAS1 oncogene (MasR; Mm00434823_s1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Mm99999915) was used as the house-keeping gene and relative expression was quantified using the $2^{\Delta\Delta Ct}$ method. Results were calculated as the percentage of the WT littermate controls.

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