Neuropharmacology xxx (2014) 1–9

Contents lists available at ScienceDirect

Neuropharmacology

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

Defects in dendrite and spine maturation and synaptogenesis associated with an anxious-depressive-like phenotype of $GABA_A$ receptor-deficient mice

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Keywords:
Neurogenesis
Hippocampal synaptogenesis
Synaptic spines
Animal model of anxious depression
Postnatal development

A B S T R A C T

Mice that were rendered heterozygous for the γ2 subunit of $GABA_A$ receptors ($\gamma2^{+/-}$ mice) have been characterized extensively as a model for major depressive disorder. The phenotype of these mice includes behavior indicative of heightened anxiety, despair, and anhedonia, as well as defects in hippocampus-dependent pattern separation, HPA axis hyperactivity and increased responsiveness to antidepressant drugs. The $\gamma2^{+/-}$ model thereby provides strong support for the GABAergic deficit hypothesis of major depressive disorder. Here we show that $\gamma2^{+/-}$ mice additionally exhibit specific defects in late stage survival of adult-born hippocampal granule cells, including reduced complexity of dendritic arbors and impaired maturation of synaptic spines. Moreover, cortical $\gamma2^{+/-}$ neurons cultured *in vitro* show marked deficits in GABAergic innervation selectively when grown under competitive conditions that may mimic the environment of adult-born hippocampal granule cells. Finally, brain extracts of $\gamma2^{+/-}$ mice show a numerical but insignificant trend ($p = 0.06$) for transiently reduced expression of brain derived neurotrophic factor (BDNF) at three weeks of age, which might contribute to the previously reported developmental origin of the behavioral phenotype of $\gamma2^{+/-}$ mice. The data indicate increasing congruence of the GABAergic, glutamatergic, stress-based and neurotrophic deficit hypotheses of major depressive disorder.

This article is part of a Special Issue entitled 'GABAergic signaling'.

1. Introduction

Molecular mechanisms that underlie the pathoetiology of major depressive disorder (MDD) remain poorly understood. However, significant evidence suggests that deficits in GABAergic transmission may play a key role in MDD. The evidence from patients pointing to compromised GABAergic transmission in MDD includes reduced brain concentrations of GABA (Sanacora et al., 1999; Hasler et al., 2007; Gabbay et al., 2012), reduced expression of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD67) (Karolewicz et al., 2010; Guilloux et al., 2012), altered expression of $GABA_A$ receptors ($GABA_A$Rs) (Merali et al., 2004; Choudary et al., 2005; Sequeira et al., 2007; Klempan et al., 2009; Klumpers et al., 2010), compromised function or loss of GABAergic interneurons (Rajkowska et al., 2007; Maciag et al., 2010; Sibille et al., 2011) and marked functional deficits in cortical inhibition (Levinson et al., 2010). Conversely, antidepressant drugs (Sanacora et al., 2002; Kucukibrahimoglu et al., 2009) and electroconvulsive therapy

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http://dx.doi.org/10.1016/j.neuropharm.2014.07.019
0028-3908/

(Sanacora et al., 2003) can normalize the reduced GABA concentrations in brain and plasma of MDD patients [for review see (Croarkin et al., 2011; Luscher et al., 2011)].

In developing neurons, GABA and $GABA_ARs$ act in concert with brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin receptor kinase (Trk)B to form an interdependent positive feedback loop that is particularly important for maturation of neuronal dendrites and GABAergic synapses (Rico et al., 2002; Chen et al., 2011; Porcher et al., 2011; Waterhouse et al., 2012). Consistent with these studies, reduced expression of BDNF and TrkB are implicated in MDD based on analyses of postmortem brain of suicide victims (Dwivedi et al., 2003, 2005; Karege et al., 2005) and depressed subjects (Tripp et al., 2012).

Mice that were rendered heterozygous for the γ2 subunit of $GABA_ARs$ ($\gamma2^{+/-}$ mice) exhibit behavioral, cognitive, neuroendocrine, and pharmacological aberrations expected of a mouse model of major depressive disorder [for review see (Luscher et al., 2011; Smith and Rudolph, 2012)]. This phenotype has a developmental origin in-between the third and fifth postnatal week of mice (Earnheart et al., 2007; Shen et al., 2012), Moreover, the phenotype of $\gamma2^{+/-}$ mice includes reduced survival of adult-born hippocampal neurons, which are thought to serve as cellular substrates of antidepressant drug action (Earnheart et al., 2007; Samuels and Hen, 2011). Adult-born neurons are also critically important for pattern separation and completion (Clelland et al., 2009; Sahay et al., 2011), a cognitive measure that is compromised in MDD (Mogg et al., 2006; Eley et al., 2008; Leal et al., 2014). In the $\gamma2^{+/-}$ model a defect in pattern separation is illustrated by a significant negative bias in an ambiguous cue discrimination task (Crestani et al., 1999).

Functional interactions between $GABA_ARs$ and BDNF in neural maturation are consistent with the neurotrophic deficit hypothesis of MDD (Duman et al., 1997; Duman and Monteggia, 2006). However, whether the phenotype of $\gamma2^{+/-}$ mice involves defects in neural maturation and synaptogenesis is not yet known. To address this issue we here have extended our analyses of adult-born hippocampal neurons in the $\gamma2^{+/-}$ model mouse. We confirm that granule cell progenitors of $\gamma2^{+/-}$ mice proliferate at normal rates. We also show that they migrate normally but then fail to survive between two and three weeks after exit from the cell cycle. Failure to survive is reflected in significant defects in dendrite and spine maturation of adult-born granule cells. Moreover, experiments in cultured neurons indicate that γ2 subunit-deficient neurons exhibit marked deficits in synaptogenesis when these neurons are grown in competition with WT neurons, i.e. conditions that are reminiscent of the competitive environment of adult-born hippocampal neurons. Lastly, we provide evidence that $GABA_AR$ deficits of $\gamma2^{+/-}$ mice may cause developmental reductions of BDNF expression that could contribute to the developmental depression-related phenotype of $\gamma2^{+/-}$ mice.

2. Experimental procedures

2.1. Production and husbandry of mice

$GABA_AR$ γ2 subunit heterozygous ($\gamma2^{+/-}$) mice used for this study were backcrossed onto the 129X1/SvJ genetic background for >40 generations (Gunther et al., 1995; Crestani et al., 1999). They were produced in our own breeding colony as littermates by crossing WT and $\gamma2^{+/-}$ mice, genotyped at the time of weaning using PCR of tail biopsies (Alldred et al., 2005) and kept on a standard 12 h:12 h light–dark cycle with food and water available ad libitum. GFP-transgenic mice (Hadjantonakis et al., 1998) carrying a transgene encoding Enhanced Green Fluorescent Protein (Clontech) driven by the ubiquitously active chicken β-actin promoter and CMV intermediate early enhancer were obtained from JAX Mice (Stock # 003116, Jackson Laboratory, Bar Harbor, ME). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Pennsylvania State University and performed in accordance with relevant guidelines and regulations of the National Institutes of Health. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize *in vitro* alternatives to *in vivo* techniques, when available.

2.2. Bromodeoxyuridine labeling and quantitation

Two different bromodeoxyuridine (BrdU) labeling protocols were used to quantify proliferation and neuronal survival of granule cells (Earnheart et al., 2007). To quantify replicating cells, three-week-old mice were injected with a single dose of BrdU of 200 mg/kg (20 mg/ml), and the brains were perfused and harvested 24 h or 48 h later. For quantification of more mature neurons, three-week-old mice were administered BrdU (4 × 80 mg/kg i.p. at 2 h intervals, in saline at 8 mg/ml, pH 7.4) and the brains were harvested either 14 d or 28 d later. The mice were anesthetized with ketamine/xylazine/acepromazine (110, 20, and 3 mg/kg, i.p.) (Schein, Melville, NY), transcardially perfused with ice-cold phosphate buffered saline (PBS), followed by 4% paraformaldehyde in PBS, postfixed for 12 h in the same solution, and cryoprotected by incubation overnight in 30% sucrose. Serial coronal sections (35 μm) through the hippocampus were cut from frozen brains with a sliding microtome. For quantitation of BrdU-labeled cells the sections were pretreated with 2 N HCl for 30 min at 37 °C and washed with 0.1 M sodium borate and PBS and immunostained with monoclonal rat anti-BrdU antibody (1:500; Accurate Chemical, Westbury, NY). For double labeling with DCX or NeuN and BrdU the sections were first stained with goat anti-DCX (1:1000; Santa Cruz Biotechnology, Dallas, TX) or mouse anti-NeuN (1:1000; Chemicon, Temecula, CA), then fixed in 4% paraformaldehyde (20 min at room temperature), treated with 2 N HCl, and stained with anti-BrdU as above. The sections were developed with Cy3-conjugated secondary anti rat (1:500; Molecular probes, Carlsbad, CA) and FITC-conjugated secondary anti guinea pig or mouse antibodies (Jackson ImmunoResearch, West Grove, PA). The number of BrdU or BrdU plus DCX/NeuN positive neurons in the subgranule and granule cell layer of confocal images was counted across sections of the entire bilateral hippocampus as described (Earnheart et al., 2007).

2.3. Analyses of migration and reconstruction and analyses of dendritic arbors

Twelve-week-old female WT and $\gamma2^{+/-}$ littermate mice were anesthetized with an overdose of Avertin [1.25% (w/v) 2,2,2-tribromoethanol in 5% 2-methyl-2-butanol) (both from Sigma–Aldrich) (375 mg/kg, 30 ml/kg i.p.)] and perfused first briefly with PBS and then with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were postfixed in the same solution for 24 h, rinsed in PBS and sectioned coronally (50 μm) using a vibratome (Vibratome; St Louis). Floating sections were stained with goat anti DCX (1:1000, Santa Cruz Biotechnology) in 2% goat serum in PBS for 48 h at 4 °C and developed with Alexa488-conjugated secondary anti-goat antibody (1:500, Molecular Probes, Carlsbad, CA) for 1 h at room temperature. Stained sections were mounted cover-slipped on glass slides and imaged using a Olympus FV1000 laser scanning confocal microscope equipped with a 40× oil objective (N.A. = 1.3). The radial position of the DCX marked cell body within the granule cell layer was recorded and assigned a position within the inner third (inner granule cell layer), center third or outer third of the granule cell layer. Optical sections covering the full-length of the dendritic tree were collected using 1-μm *z*-axis steps. For visualization of complete granule cells dendritic trees individual image stacks were superimposed digitally. Dendrites in 3D image stacks of complete dendritic arbors were traced by means of Neurolucida explorer software (MBF Bioscience, Williston, VT) and subjected to Sholl analyses to determine changes in dendritic complexity.

2.4. Analyses of synaptic spines

To visualize spines, murine Moloney leukemia virus-based CAG-GFP viral vector (Zhao et al., 2006) essentially as described (Ge et al., 2006). Briefly, GP2-293 cells (Clontech) were grown to 80% confluence in 15-cm Petri dishes using DMEM supplemented with 15% fetal calf serum and Pen/Strep. They were co-transfected with pCAG-GFP and pCMV-VSV-G (both from Addgene, Cambridge, MA) (30 μg each/plate) using the Ca_2PO_4 co-precipitation method. The media were changed 8 h later and the culture supernatant harvested and replaced 24, 36 and 48 h after the last media change. Culture supernatants were pooled and stored in 50 ml conical tubes at −80 °C. On the day of use a 40 ml aliquot of culture supernatant was thawed and centrifuged to remove cell debris (2000 rpm, 4 °C, 5 min), filtered through a 0.45 μm filter cartridge and the virus concentrated by ultracentrifugation (25,000 rpm, 4 °C, 90 min, SW 32 rotor). The virus pellets were resuspended in 10 μl sterile phosphate buffered saline to a concentration of approximately 10^8 pfu/ml. Eight-week-old female WT and $\gamma2^{+/-}$ mice were bilaterally injected with virus (0.5 μl per site) as previously described using the following coordinates relative to bregma: anteroposterior, $-0.5 \times d$ mm; lateral, −1.6 mm; ventral, −1.9 mm, with (*d*) being the distance between bregma and lambda. Two months after injection, the mice were anesthetized with an overdose of avertin (30 ml/kg), perfused and the brains postfixed and sectioned as above. Stacks of confocal optical sections of GFP fluorescence in dendritic processes were acquired at 0.5 μm intervals with an Olympus FV1000 laser scanning confocal microscope equipped with a 60× 1.42 N.A. objective and a digital zoom of 2.5. Maximum-density projections of z-stacks were used to classify spines as thin-, stubby-, or mushroom-shaped as described by Gonzalez-Burgos et al. (2000). The spine density of each dendritic fragment was calculated by dividing the manually counted number of each spine category by the length of the recorded dendritic segment (20 μm).

Please cite this article in press as: Ren, Z., et al., Defects in dendrite and spine maturation and synaptogenesis associated with an anxious-depressive-like phenotype of $GABA_A$ receptor-deficient mice, Neuropharmacology (2014), http://dx.doi.org/10.1016/j.neuropharm.2014.07.019