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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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ABSTRACT

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 the Special Issue entitled 'GABAergic Signaling in Health and Disease'.

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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(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).

2.5. Analyses of cultured cortical neurons

Cultures of cortical neurons were generated as described (Alldred et al., 2005) using mouse embryonic day 14–15 embryos produced by mating of GFP-transgenic WT and $\gamma2^{+/-}$ mice. For mixed cultures, WT and GFP-$\gamma2^{+/-}$ dissociated neurons were mixed 9:1 at the time of plating and then processed for immunostaining at DIV21 under permeabilized conditions as described (Alldred et al., 2005). Guinea pig anti-γ2 subunit (gift of J.M. Fritschy, University of Zurich, Switzerland, 1:1500), mAb GAD-6 (0.5 μg/ml; Developmental Studies Hybridoma Bank, University of Iowa, IA) and chicken anti GFP (Abcam, Cambridge, MA) were used as primary antibodies, and the stainings were developed using AlexaFluor 647 donkey anti-mouse (Molecular Probes, Eugene, OR), Cy3 donkey anti-guinea pig (Jackson ImmunoResearch) and Alexa 488 goat anti-chicken antibodies (Molecular Probes, Eugene, OR). Fluorescent images were captured and digitized with a Zeiss Axiophot 2 microscope equipped with a 40× 1.3 N.A. objective and an ORCA-100 video camera linked to an OpenLab imaging system (PerkinElmer). The density and size of immunoreactive puncta and the degree of colocalization of pre- and postsynaptic markers were quantified as described (Alldred et al., 2005; Fang et al., 2006).

2.6. Quantitation of BDNF

Extraction of BDNF protein from mouse brain homogenates was performed using a high-recovery extraction method (Szapacs et al., 2004) and used in combination with a BDNF enzyme-linked immunosorbent assay (ELISA) (Promega Co., Madison, WI) as recommended by the manufacturer. This protocol was validated by reproducible detection of a 50% reduction of BDNF in BDNF$^{+/-}$ mice (Szapacs et al., 2004). Absorbance of the colored reaction product was measured at 450 nm.

2.7. Data analysis

Simple two group comparisons were done by Mann Whitney tests or *t*-tests using GraphPad InStat version 3.00 for Macintosh, San Diego California USA. Sholl analyses of granule cell dendrites and spine density data were analyzed by repeated measures ANOVA and followed up by *t*-tests, using SPSS software (IBM). Probabilities <0.05 were considered statistically significant.

3. Results

3.1. Characterization of defects in neurogenesis of $\gamma2^{+/-}$ mice

Previous characterization of 8–12 week old $\gamma2^{+/-}$ mice revealed significantly reduced four-week-survival of adult-born hippocampal neurons (Earnheart et al., 2007). In addition, cell-specific gene knockout of the γ2 subunit in hippocampal radial glia-like astrocytes/stem cells (RGLs) revealed that tonic inhibition of these cells by γ2-containing $GABA_A$Rs inhibits their mitotic activation and dedifferentiation into neuroblast precursor cells, as well as neural cell fate choice (Song et al., 2012). To more precisely characterize the neurogenic consequences of modest $GABA_A$R reductions in the $\gamma2^{+/-}$ model we first analyzed the proliferation and survival of hippocampal neurons in greater detail. We previously had mapped the critical period for development of the anxious-depressive-like phenotype of $\gamma2^{+/-}$ mice to the third and fourth postnatal week (Shen et al., 2012). Therefore, we chose to conduct these studies in three-week old mice, a time during development when hippocampal neurogenesis is also more prolific than in adulthood. Three-week-old $\gamma2^{+/-}$ mice and WT littermate controls were metabolically labeled with BrdU, and the number of BrdU-positive cells in the subgranule cell layer was analyzed at various time intervals thereafter. The number of BrdU-positive replicating cells in the SGZ of the DG of $\gamma2^{+/-}$ mice was indistinguishable from that of WT, independent of whether the cells were quantitated 24 h ($\gamma2^{+/-}$, 1704 ± 94.9, WT, 1848 ± 125.0, $U = 9.5$, $p > 0.05$, $n = 5$ per genotype, Mann Whitney test) (Fig. 1D) or 48 h after BrdU labeling ($\gamma2^{+/-}$, 3381.6 ± 331.5; WT, 3885.6 ± 260.9, $U = 7.0$, $p > 0.05$, $n = 5$) (Fig. 1E). Thus, in contrast to the known role of γ2-containing $GABA_A$Rs in activation of RGLs that is evident upon homozygous cell-specific knock out of the γ2 gene (Song et al., 2012), the initial activation and initial proliferation of these cells is not measurably affected by a global 50% reduction of the γ2 subunit gene dosage.

To determine the developmental time point during which newborn hippocampal neurons fail to survive we analyzed the brains of BrdU-injected mice either 14 or 28 days post labeling and quantitated the number of BrdU-positive cells in the subgranular and granule cell layers that colocalized with different neuronal markers. At 14 days post labeling the number of cells that were positive for both BrdU and the immature neuron marker DCX was unaffected by genotype ($\gamma2^{+/-}$, 8190 ± 1333.88; WT, 8691 ± 730.97, $U = 8.0$, $p > 0.05$, $n = 4$, Fig. 1F), thereby indicating normal initial differentiation of granule cell precursors. However, when the mice were harvested 28 days post labeling the number of BrdU-labeled cells that were positive for NeuN was markedly reduced compared to WT littermate controls (Fig. 1G, $\gamma2^{+/-}$, 1248.0 ± 123.8; WT, 1779 ± 134.2, $U = 0.0$, $p < 0.05$, $n = 4$, Mann–Whitney, Fig. 1G). The data indicate that granule cells of $\gamma2^{+/-}$ mice fail to survive selectively during the late stage of differentiation.

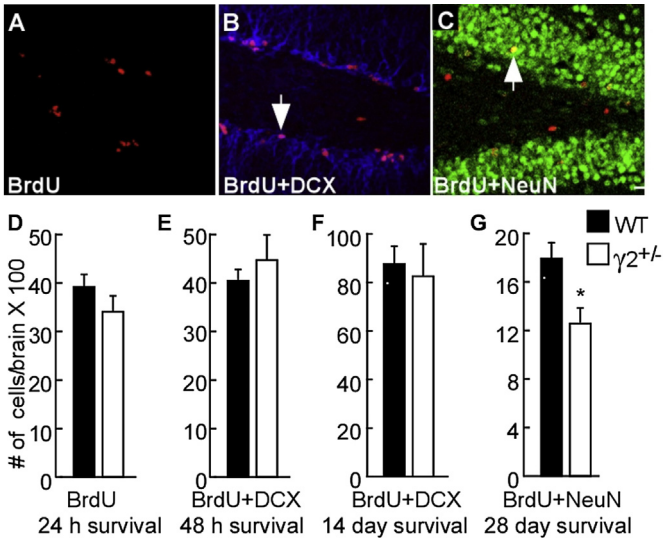

Fig. 1. Heterozygozity of the $\gamma2^{+/-}$ subunit results in a deficit in the number of NeuN positive dentate gyrus granule cell neurons born at three weeks of age. **A–C**. Representative images showing BrdU labeled brain sections of mice labeled at three weeks of age and euthanized 24 h (A), 48 h (B) or 28 d (C) after BrdU injection. Arrows indicate neurons that are doubly positive for BrdU and the immature neuronal marker DCX (B) or the mature neuronal marker NeuN (C). **D. E**. P21 mice were injected once with BrdU (200 mg/kg) and brains harvested 24 h (D) or 48 h (E) later. There was no difference in the number of BrdU positive cells in the subgranule cell layer of $\gamma2^{+/-}$ mice vs. WT controls ($p > 0.05$ for both time points). **F. G**. To label immature neurons, P21 mice were metabolically labeled (4 times 80 mg/kg every 2 h) with BrdU and the brains harvested 14 days (F) or 28 days later (G). There was no difference in BrdU/DCX double positive cells in $\gamma2^{+/-}$ mice compared to WT controls for brains harvested 14 days after BrdU injection (F) ($p > 0.05$). However, there was a significant deficit in BrdU/NeuN double positive mature neurons in $\gamma2^{+/-}$ mice analyzed 28 d post BrdU injection (G) (70.15 ± 7.0% of WT; $n = 4$ per genotype, $p < 0.05$, $U = 0$, Mann Whitney). Data indicate means ± SEM. $^*p < 0.05$, Scale bar, 20 μm.

3.2. γ2 subunit-containing $GABA_A$Rs regulate dendritic maturation

The reduction in the number of newborn $\gamma2^{+/-}$ granule cells four weeks after BrdU labeling pointed to possible defects in late stage maturation of these neurons. To test this idea we labeled immature neurons of hippocampal brain sections of 12-week-old $\gamma2^{+/-}$ and WT mice with DCX and then traced the structure of labeled dendrites in 3-D confocal image stacks using Neurolucida software. In these older mice the rate of neurogenesis is significantly lower than at three or eight weeks of age, a feature that facilitates morphological analyses of isolated dendritic trees of adult-born cells. Sholl analyses of DCX-positive granule cells revealed a significant reduction in the number of concentric circle crossings of granule cell dendrites of $\gamma2^{+/-}$ vs. WT mice [two-way ANOVA with

radial distance as within subject factor, $F(16,15) = 2.43$, $p < 0.05$, $n = 16$]. Posthoc *t*-tests revealed selective reductions in dendritic complexity in $\gamma2^{+/-}$ vs. WT mice at a distance of 70 and 90 μm from the soma ($p < 0.05$ and $p < 0.001$, respectively) and a change in the opposite direction at 290 μm from the soma ($p < 0.01$, $n = 16$) (Fig. 2A, B). In dendritic segments between 70 and 90 μm from the soma, a reduced dendritic complexity was further reflected in a reduced number of branch points of $\gamma2^{+/-}$ vs. WT neurons ($p < 0.05$, $n = 16$, *t*-test) (Fig. 2C). By contrast, the total length of dendritic trees was unaltered ($\gamma2^{+/-}$, 823.8 ± 57.6 μm, WT 829 ± 81.3 μm).

GABAergic inputs have the potential to affect the radial migration of granule cell progenitors (Ge et al., 2007a). To determine whether the phenotype of $\gamma2^{+/-}$ mice includes altered migration of granule cell progenitors we divided the granule cell layer radially into equal inner, center, and outer thirds and counted the number of DCX-positive cell bodies in each compartment/brain section and averaged these numbers across sections. DCX-positive cells were rarely found in the outer section of the granule cell layer. Moreover, the average numbers of cells in the inner and center compartments of the granule cell layer were not measurably different from WT controls ($\gamma2^{+/-}$ mice inner compartment: 88.7 ± 9.1% of WT, center compartment: 112.7 ± 57.8% of WT, $n = 3$ mice, $p > 0.5$ for both comparisons, *t*-tests).

To assess whether the maturational defect of granule cells included changes in spine density or morphology we injected the hippocampus of eight-week old $\gamma2^{+/-}$ and WT mice with a GFP-encoding retrovirus (CAG-GFP) that specifically infects replicating cells. GFP-labeled spines of adult-born neurons along dendritic segments were manually sorted into thin-, stubby- and mushroom-shaped forms (Gonzalez-Burgos et al., 2000) (Fig. 2D). Quantitation of the density of spines along dendrites revealed a significant genotype effect of $GABA_AR$ deficits on spine density (two-way ANOVA with spine type as within subject factor, $F(2, 29) = 4.3$, $p < 0.05$). Posthoc analyses indicated a selective reduction in the density of mushroom-shaped spines in $\gamma2^{+/-}$ vs. WT mice ($p = 0.014$, *t*-test) (Fig. 2E). Given the well-established correlation between density of spines and density of glutamatergic synapses the reduced spine density of $\gamma2^{+/-}$ granule cell dendrites suggests that these neurons suffer from defects in glutamatergic innervation.

3.3. $GABA_ARs$ regulate GABAergic innervation selectively under competitive conditions

Previous analyses of embryonic neurogenesis in $\gamma2^{+/-}$ and $\gamma2^{-/-}$ neurons revealed normal production, migration and survival of cortical neurons (Shen et al., 2012). Moreover, $\gamma2^{+/-}$ cortical cultures showed normal clustering of postsynaptic $GABA_ARs$ and gephyrin (Essrich et al., 1998). Similarly, conditional knock out of the $\gamma2^{+/-}$ subunit in glutamatergic neurons of the mouse cortex did not measurably affect GABAergic innervation (Schweizer et al., 2003). By contrast, sparse or mosaic homozygous knock out of the γ2 subunit *in vivo* indicated that under some conditions $GABA_ARs$ are important for normal GABAergic innervation (Li et al., 2005; Frola et al., 2013). We hypothesized that the maturational defect in hippocampal granule cells of $\gamma2^{+/-}$ mice might reflect

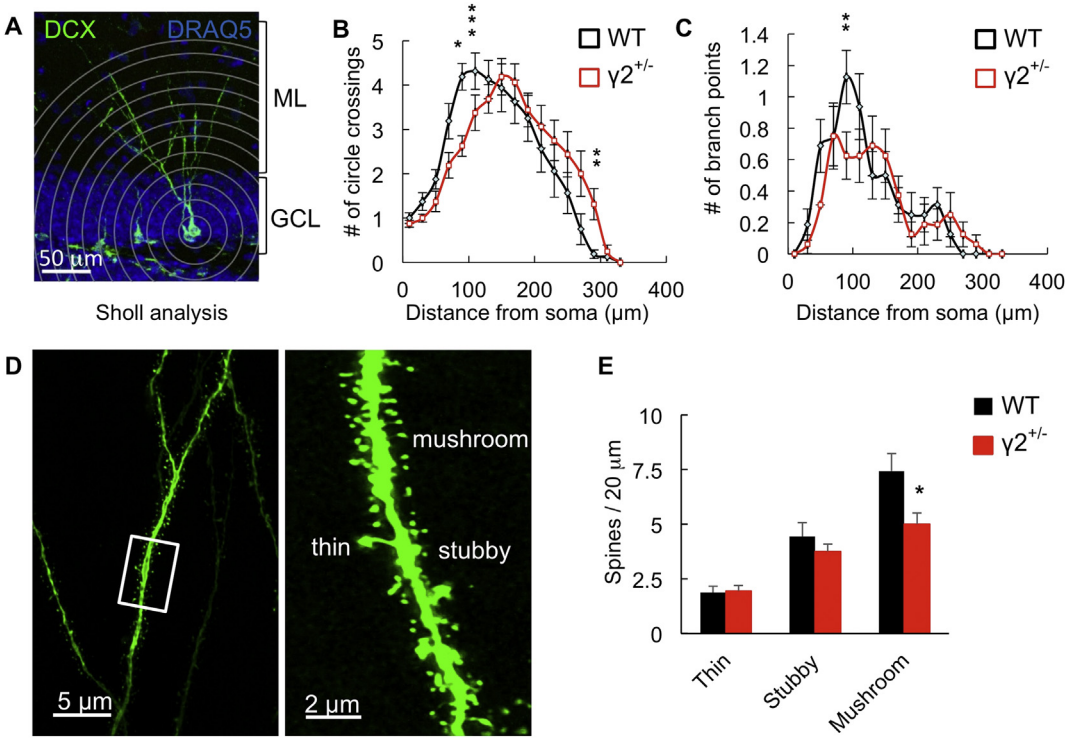

Fig. 2. Heterozygozity of the γ2 subunit results in reduced complexity of dendrites and defects in spine maturation. **A**. Example of concentric rings placed on the soma of a DCX-labeled granule cell (green) used for Sholl analysis (rings are not drawn to scale). The section was counterstained with DRAQ5 (blue) to visualize cell nuclei and demarcate the granule cell and molecular layers. ML: molecular layer. GCL: granule cell layer. **B**. Quantification of the number of circle crossings of GFP labeled granule cell dendrites of revealed a significant genotype effect [$F(16,15) = 2.4$, $p < 0.05$, $n = 16$]. Posthoc *t*-tests revealed significant reductions in dendritic complexity in $\gamma2^{+/-}$ vs. WT mice at a distance of 70 and 90 μm from the soma ($p < 0.05$ and $p < 0.001$) and a change in the opposite direction at 290 μm ($p < 0.01$, $n = 16$). **C**. There was a significant reduction in the number of branch points in $\gamma2^{+/-}$ vs. WT mice in the radial interval of 70–90 μm from the soma of granule cells ($p < 0.05$, $n = 16$, *t*-test). **D**. Representative image of a retrovirus-labeled dendritic segment of an eight-week-old granule cell. The boxed segment in the left micrograph is shown magnified on the right with thin-, stubby- and mushroom-type spines indicated. **E**. Quantification of the density of different types of spines. A two-way genotype × spine density ANOVA with spine types as within subject factors revealed a significant interaction between genotype and total spine density [$F(2, 29) = 4.3$, $p < 0.05$]. Posthoc analyses revealed a significant reduction selectively in mushroom type spines in granule cells of $\gamma2^{+/-}$ vs. WT mice ($p < 0.05$, $n = 16$, *t*-test with Bonferroni correction). Data represent means ± SEM, $^*p < 0.05$, $^{**}p < 0.01$.

competition of immature neurons with more mature neurons for proper GABAergic innervation. To examine this idea, we co-cultured GFP-transgenic $\gamma2^{+/-}$ subunit heterozygous neurons (GFP, $\gamma2^{+/-}$) with an excess of WT neurons and analyzed the density of pre- and postsynaptic markers at 21 DIV. As predicted, dendrites of GFP-tagged $\gamma2^{+/-}$ neurons showed dramatic reductions in GABAergic innervation compared to WT neurons analyzed in parallel cultures (density of GAD immunoreactive puncta on dendrites of $\gamma2^{+/-}$ neurons: 56.2 ± 4.8% of WT, $n = 13–14$ neurons, $p < 0.001$, *t*-test), along with deficits in the dendritic density of punctate immunoreactivity for postsynaptic $GABA_A$Rs (54.4 ± 2.7% of WT, $p < 0.001$) (Fig. 3A, B). Interestingly, the size of γ2 immunoreactive puncta was unchanged (101.2 ± 7.3% of WT, $p > 0.05$), whereas the size of GAD puncta was significantly increased in $\gamma2^{+/-}$ neurons of co-cultures (130.5 ± 9.3% of WT, $p < 0.001$) (Fig. 3C), pointing to a presynaptic homeostatic compensatory mechanism. There was also a small decrease in the degree of colocalization in the punctate stainings for GAD and $GABA_A$R in $\gamma2^{+/-}$ vs. WT neurons (Fig. 3D). Similar defects in GABAergic innervation were seen when untagged $\gamma2^{+/-}$ neurons were co-cultured with an excess of GFP-tagged WT neurons (not shown). We conclude that γ2-containing $GABA_A$Rs contribute to normal GABAergic innervation, a property that is only evident under conditions where GABAergic axons are forced to choose among target neurons that differ in the level of expression of γ2-containing $GABA_A$Rs.

3.4. Developmental effects of $GABA_A$R deficits on BDNF expression

In developing neurons, BDNF functions as part of a positive feedback loop that promotes the endocytic stability of $GABA_A$Rs in the plasma membrane (Porcher et al., 2011). Moreover, this mechanism was recently reported to involve BDNF/TrkB-mediated Tyr phosphorylation of the γ2 subunit (Vithlani et al., 2013). In turn, $GABA_A$R-mediated neural depolarization of immature neurons activates a Ca^{2+}-dependent signaling cascade that promotes BDNF gene expression and BDNF release (Baldelli et al., 2002; Obrietan et al., 2002; Porcher et al., 2011). BDNF/TrkB signaling also promotes the postsynaptic accumulation of PSD95 and thereby contributes to maturation of dendritic synaptic spines (Yoshii et al., 2011). These data predict that the dendritic maturational defects seen in the dentate gyrus of $\gamma2^{+/-}$ mice might involve secondary reductions in BDNF expression that may amplify detrimental consequences of $GABA_A$R functional deficits. To test this idea we measured BDNF protein expression in frontal cortex, hippocampus and brain stem of one-, three- and nine-week-old female $\gamma2^{+/+}$ and $\gamma2^{+/-}$ mice. We found prominent differences in BDNF expression across postnatal development and brain regions, as expected (Fig. 4A–C). No genotype effects on BDNF expression were evident in samples from individual brain regions across all three developmental stages ($p > 0.05$ for all *t*-tests). However, a genotype × brain region ANOVA of data from three-week-old mice normalized to WT values revealed a strong trend towards lower BDNF protein levels in

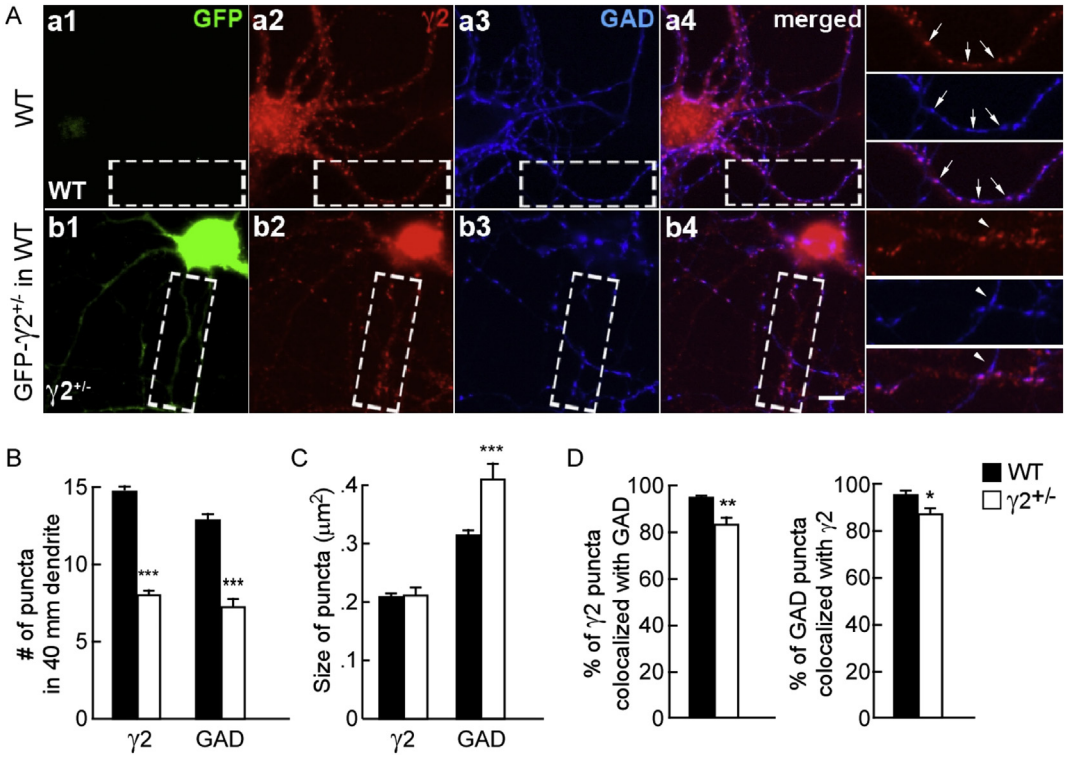

Fig. 3. Subtle deficits in γ2-containing $GABA_A$Rs result in deficits of GABAergic innervation. **A**, Cortical neurons (DIV21) derived from WT neurons were either cultured alone (top row) or mixed together with cortical neurons from GFP-transgenic $\gamma2^{+/-}$ embryos (bottom row, 9:1 excess of WT neurons). The neurons were immunostained for the γ2 subunit (red, **a2, b2**) and GAD (blue, **a3, b3**). Merged red and blue channels are shown in panels (**a4**) and (**b4**). Boxed dendritic segments are enlarged in separate panels on the right. Arrows in enlarged panels point to a WT axon(s) that faithfully grows along a WT dendrite. By contrast, arrowheads show an axon of presynaptic WT neurons that grows across the dendrite of a $\gamma2^{+/-}$ neuron, and failing to adhere to the dendrite (**a**). Mutant neurons ($\gamma2^{+/-}$) were identified by the transgene-encoded GFP fluorescence (green, **b1**). **B**. When $\gamma2^{+/-}$ neurons were co-cultured in the presence of an excess of WT neurons the density of immunoreactive puncta for γ2 (54.4 ± 2.7% of WT, $n = 13–14$ neurons, $p < 0.001$, *t*-test) and GAD (56.2 ± 4.8% of WT, $n = 13–14$, $p < 0.001$, *t*-test) along their dendrites was significantly reduced compared to WT neurons grown in pure cultures. **C**. The size of γ2 puncta was unchanged (101.2 ± 7.3% of WT, $p > 0.05$, *t*-test), whereas the size of GAD puncta (of WT presynaptic interneurons) was significantly increased when $\gamma2^{+/-}$ neurons were co-cultured with an excess of WT neurons (130.5 ± 9.3% of WT, $p < 0.001$), suggesting presynaptic compensation for limiting amounts of postsynaptic $GABA_A$Rs. **D**. The number of γ2 puncta colocalized with GAD in $\gamma2^{+/-}$ neurons co-cultured with WT was reduced (87.7 ± 3.62% of WT; $n = 13–14$, $p < 0.01$, *t*-test). Similarly, the number of GAD puncta colocalized with γ2 was reduced in $\gamma2^{+/-}$ neurons co-cultured with WT (91.4 ± 2.8% of WT; $n = 13–14$, $p < 0.05$). Data represent means ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

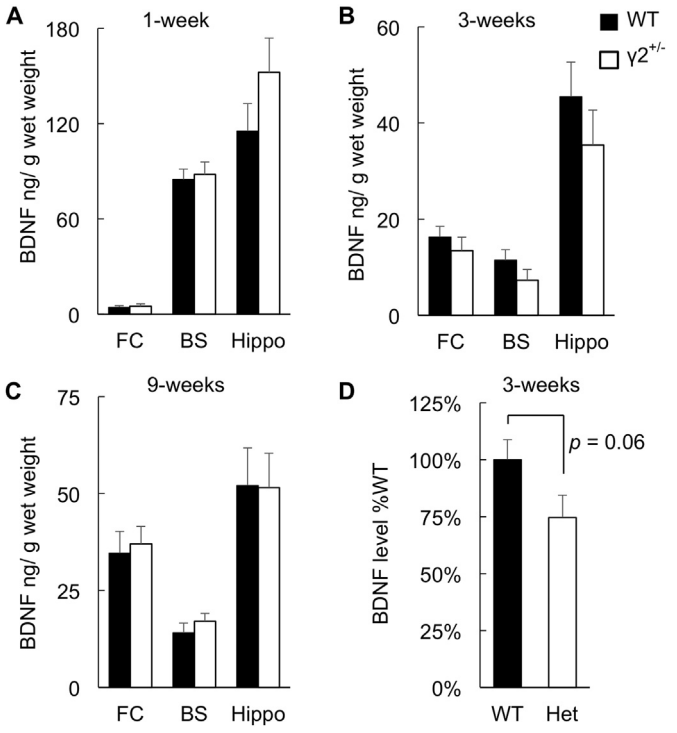

Fig. 4. BDNF protein levels in different brain regions across postnatal development. **A**–**C**. BDNF protein levels were measured in extracts from frontal cortex (FC), brain stem (BS) and hippocampus (Hippo) of one-, three-, and nine-week-old $\gamma2^{+/-}$ and WT littermate mice. No differences in BDNF expression were observed in any of the three brain regions analyzed (*t*-tests, $p > 0.05$ for all comparisons). **D**. However, a genotype × brain region ANOVA of data from three-week-old mice normalized to WT revealed a strong trend towards lower BDNF protein expression in $\gamma2^{+/-}$ vs. WT mice [$F(1, 34) = 3.72$, $p = 0.06$, $n = 18$]. BDNF levels are reported as ng BDNF/g wet weight tissue ± SEM.

$\gamma2^{+/-}$ vs. WT mice [$F(1, 35) = 3.72$, $p = 0.06$, $n = 18$)] (Fig. 4D). We previously showed that the anxious depression-related increase in emotional reactivity of $\gamma2^{+/-}$ mice has a developmental origin between the third and end of fourth postnatal week (Earnheart et al., 2007; Shen et al., 2012). The data suggest that developmental brain-region-independent reductions in BDNF expression might contribute to the known behavioral abnormalities of $\gamma2^{+/-}$ mice.

4. Discussion

We previously reported that the γ2 subunit of $GABA_A$Rs is critically important for the survival of adult-born neurons of the hippocampus (Earnheart et al., 2007). We here have extended these findings and show that similarly compromised neurogenesis is also present in juvenile mice. Moreover, we show that neurons fail to survive at a relatively late stage of differentiation, at a time when they no longer express the immature marker DCX. Loss of neurons was associated with a defect of dendritic arbor maturation in the molecular layer proximal to the granule cell layer. Impaired differentiation of granule cells overlaps temporally with a period of enhanced synaptic plasticity of adult born granule cells (Ge et al., 2007b) that is thought to be pivotal for pattern separation and completion (Sahay et al., 2011), a hippocampus-dependent form of cognitive disambiguation of similar life situations (Schmidt et al., 2012). Consistent with defects in pattern separation, $\gamma2^{+/-}$ mice show selective defects in ambiguous cue discrimination learning while performing normally or better than WT mice in other hippocampus-dependent learning and memory tasks (Crestani et al., 1999). Deficits in the resolution of ambiguity reminiscent of those of $\gamma2^{+/-}$ mice are commonly associated with MDD (Austin et al., 1999; Schatzberg et al., 2000; Rogers et al., 2004; Anda et al., 2006), suggesting that defects in granule cell maturation may contribute to the anxious-depressive phenotype of $\gamma2^{+/-}$ mice.

In addition to dendritic maturation, the γ2 subunit of $GABA_A$Rs is known to be essential for GABA-mediated inhibition of RGL activation, and for the control of neural vs. glial cell fate choice of RGL-derived neural progenitors (Song et al., 2012). This was shown by cell type specific knock out of the γ2 subunit in RGLs, which results in their rapid dedifferentiation and mitotic activation. Increased proliferation of neural precursor cells was similarly observed upon deletion of the α4 subunit (but not the δ subunit) of $GABA_A$Rs (Duveau et al., 2011), indicating that the receptors that mediate the tonic GABA input that inhibits activation of RGLs likely have an α4βγ2 subunit composition. However, we showed here that heterozygous deletion of the γ2 subunit did not measurably affect this mechanism, as evidence by normal numbers of BrdU labeled cells 24 and 48 h after labeling. This is consistent with evidence that the γ2-subunit is largely dispensable for assembly of αβ receptor complexes, and that αβ complexes can form GABA-gated channels in non-synaptic membranes, albeit with significantly lower channel conductance than αβγ2 receptors (Baer et al., 1999; Lorez et al., 2000; Mortensen and Smart, 2006). By contrast, the γ2 subunit is absolutely essential for accumulation of $GABA_A$Rs at synapses, the typical function of γ2-containing $GABA_A$Rs in mature neurons (Essrich et al., 1998; Schweizer et al., 2003). In four-week-old granule cells the γ2 subunit is thought to be part of α2βγ2 receptors that are invariably enriched at synapses. This view is supported by analyses of knockout mice lacking the α2 subunit, which show selective maturational defects of granule cells comparable to those of $\gamma2^{+/-}$ mice (Duveau et al., 2011).

Sparse knock down of the γ2 subunit by in utero electroporation and analyses of mice with a mosaic γ2 loss of function allele have indicated that $GABA_A$Rs play a role in GABAergic synapse formation (Li et al., 2005; Frola et al., 2013). Defects in GABAergic innervation were also observed following sparse shRNA-mediated knockdown of $GABA_A$R interacting trafficking proteins (Fang et al., 2006; Yuan et al., 2008). This was unexpected given that GABAergic innervation is unaffected in γ2 knock-out mice and primary cultured neurons (Essrich et al., 1998). However, we now show that even a partial reduction in the expression of the γ2 subunit results in a marked reduction in GABAergic innervation when $\gamma2^{+/-}$ neurons are grown in competition with WT neurons. This competitive environment explains the apparent discrepancies above between phenotypes observed following sparse vs. global knock out of the γ2 subunit. Moreover, this situation is reminiscent of the condition faced by developing adult-born granule cells that have to compete with mature neurons for GABAergic innervation. Consistent with a direct role of GABAergic innervation $GABA_A$Rs overexpressed in heterologous cells can initiate GABAergic innervation when co-cultured with neurons (Fuchs et al., 2013). However, this synaptogenic effect of $GABA_A$Rs is substantially weaker than that of $GABA_A$Rs co-expressed with the synaptic cell adhesion protein neuroligin-2 (Dong et al., 2007).

The deficits in granule cell maturation seen in $\gamma2^{+/-}$ mice are reminiscent of similar deficits seen in multiple mouse lines with genetically induced defects in BDNF/TrkB signaling (Sairanen et al., 2005; Kaneko et al., 2012; Waterhouse et al., 2012). This is consistent with substantial evidence that neurotrophic mechanisms of BDNF/TrkB are intrinsically intertwined with mechanisms of GABAergic transmission. First, BDNF functions upstream of $GABA_A$Rs by enhancing the relative excitability of GABAergic versus glutamatergic neurons (Wardle and Poo, 2003) and by promoting the release of GABA (Jovanovic et al., 2000) from parvalbumin-

positive interneurons (Waterhouse et al., 2012), a mechanism that delimits the mitotic activation of RGLs (Song et al., 2012). Second, BDNF/TrkB signaling serves to stabilize $GABA_AR$s at the cell surface, both in immature (Porcher et al., 2011) and mature neurons (Vithlani et al., 2013). In immature neurons, BDNF/TrkB facilitates $GABA_AR$ cell surface accumulation (Porcher et al., 2011) and hence GABA/$GABA_AR$ mediated membrane depolarization, which enables Ca^{2+} entry by V-gated Ca^{2+} channels (Maric et al., 2001; Borodinsky et al., 2003; Fiszman and Schousboe, 2004; Schmidt-Hieber et al., 2004; Gascon et al., 2006) and NMDARs (Tashiro et al., 2006), followed by activation of diverse Ca^{2+} dependent Ser/Thr kinases that promote CREB phosphorylation and CREB-dependent gene transcription (Shaywitz and Greenberg, 1999; Nakagawa et al., 2002; Fujioka et al., 2004; Gur et al., 2007; Jagasia et al., 2009). In addition, $GABA_AR$-mediated membrane depolarization facilitates BDNF release (Porcher et al., 2011). Moreover, one of the most prominent target genes activated by CREB is BDNF (Shieh et al., 1998; Tao et al., 1998; Obrietan et al., 2002). These mechanisms indicate that BDNF functions not only upstream but also downstream of GABAergic transmission. Consistent with GABAergic control of BDNF expression and release we found a near significant trend ($p = 0.06$) for reduced BDNF protein expression in developing $\gamma2^{+/-}$ vs. WT mice. Our analyses of genotype dependent changes in individual brain regions were likely underpowered due to dynamic, behavioral state- and brain region-dependent variation in BDNF expression. Nevertheless, our data strongly suggest that BDNF expression is regulated by GABAergic transmission *in vivo*. Therefore, it is conceivable that reduced BDNF expression and function contributes to the postnatal developmental origin of the anxious-depressive-like phenotype of $\gamma2^{+/-}$ mice (Earnheart et al., 2007; Shen et al., 2010, 2012). Although far from detectable by biochemical means, it is likely that reduced BDNF transcription also applies to immature granule cells of $\gamma2^{+/-}$ mice. These neurons are likely more vulnerable than embryo-derived neurons as they need to compete for GABAergic innervation with mature surrounding neurons. Consistent with this interpretation, we showed previously that the rate of proliferation, migration and survival of embryonic cortical neurons is unaffected in $\gamma2^{+/-}$ and $\gamma2^{-/-}$ mice (Shen et al., 2012).

In addition to a deficit in dendritic complexity and indirect evidence for reduced GABAergic innervation, granule cells of $\gamma2^{+/-}$ mice showed a reduction in the dendritic density of mushroom type synaptic spines. Mushroom type spines are known to serve as sites of stable and functionally mature glutamatergic innervation (Matsuzaki et al., 2001; Ashby et al., 2006). Thus, the dendritic spine abnormalities of $\gamma2^{+/-}$ mice suggest association of functional defects in glutamatergic transmission with the established anxious-depressive phenotype of these mice. Atrophy of dendrites and spines are hallmarks also of stress-based, glutamatergic (Popoli et al., 2012) and neurotrophic deficit hypotheses of MDD (Duman and Li, 2012). In sum, our findings reported here suggest increasing congruence of all these hypotheses with the GABAergic deficit hypothesis of MDD (Luscher et al., 2011).

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