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Synaptic Localization of α5 GABA (A) Receptors via Gephyrin Interaction Regulates Dendritic Outgrowth and Spine Maturation

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Abstract

$GABA_A$ receptor subunit composition is a critical determinant of receptor localization and physiology, with synaptic receptors generating phasic inhibition and extrasynaptic receptors producing tonic inhibition. Extrasynaptically localized α5 $GABA_A$ receptors are largely responsible for tonic inhibition in hippocampal neurons. However, we show here that inhibitory synapses also contain a constant level of α5 $GABA_A$ receptors throughout neuronal development, as measured by its colocalization with gephyrin, the inhibitory postsynaptic scaffolding protein. Immunoprecipitation of the α5 subunit from both cultured neurons and adult rat brain coimmunoprecipitated gephyrin, confirming this interaction *in vivo*. Furthermore, the α5 subunit can interact with gephyrin independent of other synaptically localized alpha subunits, as shown by immunoprecipitation experiments in HEK cells. By replacing the α5 predicted gephyrin binding domain (Residues 370–385) with either the high affinity gephyrin binding domain of the α2 subunit or homologous residues from the extrasynaptic α4 subunit that does not interact with gephyrin, α5 $GABA_A$ receptor localization shifted into or out of the synapse, respectively. These shifts in the ratio of synaptic/extrasynaptic α5 localization disrupted dendritic outgrowth and spine maturation. In contrast to the predominant view of α5 $GABA_A$ receptors being extrasynaptic and modulating tonic inhibition, we identify an intimate association of the α5 subunit with gephyrin, resulting in constant synaptic levels of α5 $GABA_A$R throughout circuit formation that regulates neu ronal development.

Keywords

alpha 5 GABA(A)R; gephyrin; spine morphology; dendritic outgrowth

INTRODUCTION

Gamma-aminobutyric acid type A receptors ($GABA_A$Rs) are pentameric chloride permeable ligand gated ion channels mediating GABAergic inhibition in the brain. Synaptic release of GABA activates postsynaptically localized $GABA_A$Rs to trigger fast inhibitory currents, while ambient GABA results in activation of extrasynaptic receptors and generation of tonic inhibition. The subunit composition of the receptor, typically two α subunits, two β subunits,

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and either a γ or a δ subunit, determines receptor cell surface localization as well as physiological and pharmacological properties. Receptors containing α1, α2, and/or α3 subunits are primarily located at GABAergic synapses via direct association with gephyrin, the key inhibitory postsynaptic scaffolding protein responsible for $GABA_AR$ and glycine receptor localization (Tyagarajan and Fritschy, 2014). $GABA_ARs$ containing the α4 and α6 subunits do not interact with gephyrin, are extrasynaptically localized, and are responsible for tonic GABA currents (Glykys and Mody, 2007). The α5 subunit assembles with β and γ subunits, is reported to be primarily localized extrasynaptically (Brunig et al., 2002; Christie and de Blas, 2002), and is primarily responsible for generating tonic inhibition in the hippocampus (Glykys et al., 2008). Extrasynaptically, the α5 subunit colocalizes with radixin, a cytoskeletal protein linking actin to the plasma membrane (Loebrich et al., 2006).

The α5 subunit is suggested to play an important role in hippocampal pyramidal neuron development (Marchionni et al., 2007; Giusi et al., 2009). However, the contribution of α5-containing $GABA_AR$ localization to neuronal development is unknown, and the mechanism for synaptic accumulation of the α5 subunit has not been established. We show here that surface α5 $GABA_ARs$ significantly colocalize with gephyrin, with α5 synaptic content being maintained at a constant ratio throughout development. We then demonstrate that the α5 subunit directly interacts with gephyrin, and that disruption of Residues 370–385 of the α5 subunit alters both α5 association with gephyrin and receptor synaptic/extrasynaptic localization, but is not required for cluster formation or maintenance of surface receptor number. Finally, we show that balanced distribution of the α5 subunit at synaptic and extrasynaptic sites is important for proper dendritic outgrowth and spine maturation. Together these results support a synaptic role in neuronal development for the α5 $GABA_AR$ subtype.

METHODS

DNA Constructs and Antibodies

The $\alpha5^{WT}$, $\alpha5^{\alpha4GBD}$, $\alpha5^{\alpha2GBD}$, and RFP (red monomeric fluorescent protein mCherry) tagged gephyrin construct were generated by PCR cloning (Li et al., 2011) from CDNAs (kindly provided by Stephen J. Moss). The pH-sensitive GFP tagged α2 $GABA_AR$ subunit ($\alpha2^{WT}$) has been described previously (Tretter et al, 2008). All constructs were fully sequenced. The following antibodies were used: rabbit anti-α5 (Cat # 224503), mouse anti-gephyrin (RRID: AB_887719), rabbit anti-VGAT (RRID: AB_887871), mouse anti-α1 (RRID: AB_10597955), and rabbit anti-α2 (RRID: AB_2108839; Synaptic Systems, Gottingen, Germany); rabbit anti-radixin (Cat # R3653, Sigma, St. Louis, MO); chicken anti-GFP (RRID: AB_10000240, Aves Labs, Tigard, OR); rabbit anti-GFP (RRID: AB_221570, Life Technologies, Carlsbad, CA); mouse anti-RFP (RRID: AB_1141717, Abcam, Cambridge, MA) and secondary antibodies for immunofluorescence (goat anti-rabbit Alexa Fluor 568 RRID: AB_143011; goat anti-rabbit Alexa Fluor 488 RRID: AB_10562715; Goat anti-mouse Alexa Fluor 647 RRID: AB_141725; goat anti-chicken Alexa Fluor 488 Cat # 11309, Life Technologies, Carlsbad, CA). Rabbit IgG (RRID: AB_737197), mouse IgG (RRID: AB_737182), and goat anti-α5 (RRID: AB_2109314) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Transfection

All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Hippocampal neurons were prepared from embryonic day 18 rats and nucleofected (Lonza) at plating (Jacob et al., 2005). HEK-293 cells were also transfected using nucleofection.

Immunofluorescence, Imaging, and Analysis of Cells

Surface $GABA_AR$ $\alpha5$ or anti-GFP staining was done under nonpermeabilized conditions, followed by permeabilization and staining for $\alpha5$, GFP, and/or gephyrin as previously described (Jacob et al., 2005). Images were taken on a Nikon A1 Confocal microscope using a 60× oil immersion objective (NA 1.49) at a 2× zoom. Samples for confocal imaging were sequentially scanned with individual lasers (488, 561, and 640 nm) and an appropriate emission band pass filter (500–550 and 575–625 nm) or long pass filter (650LP) to avoid any spectral bleed-through between channels. Data were analyzed using NIS Elements software (Nikon, NY), with colocalization determined as previously described (Tretter et al., 2008). In the gephyrin and $\alpha5$ $GABA_AR$ colocalization studies, 20-μm length regions (ROIs) from two to three proximal dendrites per neuron were used for analysis. For comparison between $\alpha5^{WT}$, $\alpha5^{\alpha4GBD}$, and $\alpha5^{\alpha2GBD}$, the researcher was blinded to the experimental conditions. For cluster analysis, thresholding was kept constant between cultures. For total $\alpha5$ surface fluorescence measurements (both clustered and diffuse receptors), fluorescence values in the same ROIs were measured without thresholding. 3D reconstructions of confocal z series acquired with a 40× oil immersion objective, 1× zoom, were used for Sholl analysis, and analyzed using ImageJ software. Spine morphology studies were performed as previously described (Jacob et al., 2009). Briefly, images were analyzed from 3D reconstructions of confocal z series acquired with a 60× oil immersion objective using a 4× zoom. Spine length was determined using NIS Elements by measuring the length from the spine head to where the spine intersected the dendritic shaft. We classified spines as either mushroom or filopodia, with a mushroom spine having a spine head twice the size of the spine neck, and the remaining spines classified as filopodia.

Immunoprecipitation and Western Blots

For immunoprecipitation from hippocampal neuronal cultures, neurons were nucleofected with $\alpha5^{WT}$ at plating. At *DIV* 14, cells were lysed in Buffer A containing 50 m*M* Tris-HCl (pH 7.6), 50 m*M* NaCl, 1 m*M* EDTA, 0.25% Igepal, 10 m*M* NaF, 2 m*M* sodium orthovanadate, and protease inhibitor cocktail (Sigma, St. Louis, MO), solubilized for 15 min at 4°C, and spun at 1,000*g* for 10 min. Extracts were then immunoprecipitated with either rabbit anti-GFP (5 μg), or rabbit IgG as a negative control. For hippocampal and cortical rat tissue, adult female rats were killed using CO_2 followed by cervical dislocation. The hippocampus and cortex were dissected and homogenized as previously described (Brady et al., 2013), diluted approximately fivefold in Buffer A, and immunoprecipitated with rabbit anti-$\alpha5$ antibody crosslinked to Protein A Sepharose beads with BS^3 (Thermo Scientific, Rockford, IL). The beads were eluted by incubating beads twice with 50 m*M* glycine for 5 min, as previously described (Sousa et al., 2011), and SDS-PAGE loading buffer was added. For immunoprecipitation from HEK cells, HEK cells were transfected

with RFP-gephyrin, an untagged β3 subunit and either a $\alpha 2^{WT}$ or $\alpha 5^{WT}$ construct, and lysates were immunoprecipitated with rabbit anti-GFP as described above.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (San Diego, CA) on 3–4 independent cultures, 12–18 neurons per culture, using two way ANOVA with Tukey *post hoc* test for dendritic morphology analysis or one-way ANOVA with paired t-test post-hoc tests for all other studies. All values are given as mean ± standard error of the mean.

RESULTS

Inhibitory Synapses Contain a Constant Level of α5-Containing $GABA_A$Rs Throughout Development

$GABA_A$Rs containing the α5 subunit are generally considered to be extrasynaptic; however, previous studies have found some evidence for synaptic localization (Christie and de Blas, 2002; Serwanski et al., 2006). We first examined whether a pool of endogenous α5 $GABA_A$Rs was synaptically located and if receptor localization changes throughout development. Hippocampal neurons were fixed and stained for surface α5 and gephyrin, the postsynaptic inhibitory scaffold protein (Jacob et al., 2005; Flores and Mendez, 2014; Tyagarajan and Fritschy, 2014). Analysis of the number of gephyrin clusters colocalized with α5 found a high level of colocalization, with approximately a 60% association maintained at a uniform level throughout development [DIV *7* 62.67 ± 7.38, DIV *14* 53.34 ± 4.62, DIV *21* 72.34 ± 9.06; Fig. 1(A,C)]. These data support a role for synaptically located α5 $GABA_A$Rs contributing to GABAergic inhibition, in addition to the established role of extrasynaptic α5 $GABA_A$Rs.

To further investigate mechanisms of α5 $GABA_A$R synaptic localization, we then introduced a myc tag and a pH-sensitive GFP variant at the N-terminus of the α5 subunit ($\alpha 5^{WT}$). It has been shown that insertion of these tags into other $GABA_A$R subunits are functionally silent (Jacob et al., 2005; Tretter et al., 2008, 2011; Mukherjee et al., 2011). To confirm that these tags do not induce major alterations, we examined the total number of surface α5 containing $GABA_A$R clusters and the total number of gephyrin clusters between nontransfected and transfected neurons at all developmental stages [Fig. 1(A,B,D)]. We found no significant difference between groups, indicating that $GABA_A$Rs containing tagged α5 subunits do not induce changes in the development of GABAergic synapses. We then examined synaptic localization of the $\alpha 5^{WT}$ construct. Similar to nontransfected neurons, the number of gephyrin clusters associated with $\alpha 5^{WT}$ remains constant throughout development [DIV *7* 59.24 ± 7.95, DIV *14* 59.25 ± 7.25, DIV *21* 62.76 ± 6.45; Fig. 1(B,C)]. As the α5 subunit has been shown to associate with radixin in the extrasynaptic membrane at DIV 13–14 (Loebrich et al., 2006), surface $\alpha 5^{WT}$ colocalization with radixin was also assessed: there was a significant decrease in association of radixin and α5 between DIV 7 and DIV 21 (DIV *7* 70.45 ± 10.12%, DIV *21* 26.60 ±9.05%, $p = 0.0270$; Supporting Information Fig. S1). These data suggest that during this developmental period, while α5-radixin association decreases over time, the number of synapses (as represented by gephyrin) with α5-containing $GABA_A$Rs remains constant. In summary, these data show that a major portion of

α5 GABA$_A$Rs is observed at synaptic locations throughout the key period of synaptogenesis and in an established hippocampal neuron culture circuit.

α5-Containing GABA$_A$R and Gephyrin Are Intimately Associated in Cultured Neurons and *In Vivo*

To further demonstrate a close association of α5 with gephyrin in neurons, we performed immunoprecipitations on hippocampal neuron cultures transfected with the α5^{WT} construct. Immunprecipitation of the tagged α5 subunit with an anti-GFP antibody also coimmunprecipitated gephyrin [Fig. 2(A)]. To confirm that α5 and gephyrin interact *in vivo*, endogenous α5 was immunoprecipitated from adult rat hippocampal and cortical tissue, and the association of gephyrin was determined. As shown in Figure 2(B), the α5 subunit coimmunoprecipitated gephyrin, confirming that these two proteins are associated *in vivo*.

The α5 Subunit Intracellular Domain Is Sufficient for Gephyrin Binding

Our data indicate that the α5 subunit is associating with gephyrin, although this could occur solely via a direct interaction of the α5 subunit with gephyrin (via the α5 intracellular domain), or via α5 interactions with other proteins capable of directly interacting with gephyrin, such as another α subunit. A potential weak direct interaction between the α5 intracellular loop and gephyrin was suggested by minimal binding in a GST overlay assay using an α5 intracellular loop GST-fusion protein overlaid with ^{35}S-methionine labeled gephyrin (Mukherjee et al., 2011). To further examine the mechanism for α5 GABA$_A$R synaptic localization with a complete α5 subunit, we cotransfected the α5^{WT} and an RFP-gephyrin construct into HEK cells, along with an untagged β3 construct (surface expression of functional GABA$_A$Rs requires a β subunit; Connolly et al., 1996). As a positive control, a pH-sensitive GFP tagged α2^{WT} subunit was cotransfected with RFP-gephyrin and the β3 subunit in a separate set of HEK cell experiments. In neurons the RFP-gephyrin exhibits equivalent subcellular localization as endogenous gephyrin, being highly colocalized with synaptic α2 GABA$_A$R and closely apposed to the presynaptic inhibitory terminal maker VGAT [vesicular GABA/glycine transporter; Fig. 2(C,D)], indicating its ability to interact with GABA$_A$Rs. Immunoprecipitation of HEK lysates with anti-GFP resulted in coimmunoprecipitation of RFP-gephyrin by both α2^{WT} and α5^{WT} [Fig. 2(E)], indicating that these proteins are associated in a heterologous system, supporting a direct interaction between the α5 and gephyrin rather than via α1 or α2 subunits.

The α5 Gephyrin Binding Domain Contributes to Synaptic Localization of α5 GABA$_A$R

GABA$_A$R α1, α2, and α3 subunits localize to the synapse via interaction with gephyrin through specific domains in the subunit intracellular loops between transmembrane Domains 3 and 4 (Tretter et al., 2008, 2011; Mukherjee et al., 2011), whereas extrasynaptic α4 or α6 GABA$_A$Rs do not colocalize or interact with gephyrin (Sun et al., 2004; Kralic et al., 2006). To determine if the α5 sequence (Residues 370–385) homologous to the gephyrin binding domains in other alpha subunits enables α5 interaction with gephyrin, we created two different α5 chimeras; one in which the predicted gephyrin binding domain (GBD; Mukherjee et al., 2011) of α5 was replaced with the homologous region from α4 (α5$^{\alpha 4GBD}$) and another in which the α5 GBD was replaced with the α2 GBD [α5$^{\alpha 2GBD}$; Fig. 3(A)]. Chimeric GABA$_A$R expression levels, assembly and surface trafficking were equivalent to

$\alpha5^{WT}$ in HEK cells (Supporting Information Fig. S2). We then transfected neurons with the $\alpha5^{WT}$, $\alpha5^{\alpha4GBD}$, or $\alpha5^{\alpha2GBD}$ constructs and analyzed surface $GABA_AR$ $\alpha5$ subunit clustering and synaptic localization at DIV 14. In neurons expressing the chimeric receptors, both surface tagged $\alpha5$ $GABA_AR$ clusters and gephyrin overall cluster attributes were unchanged, indicating normal synaptogenesis: the total number and sum intensity of clusters were not significantly different [Fig. 3(C,D,F,G)]. In addition, the total surface fluorescence of $\alpha5$ (both clustered and diffuse), was unchanged between groups, indicating no apparent change in receptor trafficking [Fig. 3(E)]. We found a significant decrease in surface receptor colocalization with gephyrin in neurons transfected with $\alpha5^{\alpha4GBD}$ compared to $\alpha5^{WT}$ and $\alpha5^{\alpha2GBD}$, and a significant increase in colocalization in neurons transfected with $\alpha5^{\alpha2GBD}$ compared to $\alpha5^{WT}$ [$\alpha5^{WT}$ 60.80 ± 3.16%; $\alpha5^{\alpha4GBD}$ 45.29 ± 2.64%; $\alpha5^{\alpha2GBD}$ 74.68 ± 5.06%; $\alpha5^{WT}$ vs. $\alpha5^{\alpha4GBD}$ $p = 0.0055$, $\alpha5^{WT}$ vs. $\alpha5^{\alpha2GBD}$ $p = 0.048$, $\alpha5^{\alpha4GBD}$ vs. $\alpha5^{\alpha2GBD}$ $p = 0.0012$, Fig. 3(B,H)]. Gephyrin clusters colocalized with $\alpha5$ also exhibited corresponding changes: cluster sum area [$\alpha5^{WT}$ 0.54 ± 0.045; $\alpha5^{\alpha4GBD}$ 0.32 ± 0.046; $\alpha5^{\alpha2GBD}$ 1.15 ± 0.063; $\alpha5^{WT}$ vs. $\alpha5^{\alpha4GBD}$ $p = 0.0096$, $\alpha5^{WT}$ vs. $\alpha5^{\alpha2GBD}$ $p = 0.0002$, $\alpha5^{\alpha4GBD}$ vs. $\alpha5^{\alpha2GBD}$ $p < 0.0001$, Fig. 3(B,I)] and sum intensity normalized to control [$\alpha5^{WT}$ 100; $\alpha5^{\alpha4GBD}$ 56.89 ± 7.52; $\alpha5^{\alpha2GBD}$ 189.10 ± 31.81; $\alpha5^{WT}$ vs. $\alpha5^{\alpha4GBD}$ $p = 0.0004$, $\alpha5^{WT}$ vs. $\alpha5^{\alpha2GBD}$ $p = 0.0086$, $\alpha5^{\alpha4GBD}$ vs. $\alpha5^{\alpha2GBD}$ $p = 0.002$, Fig. 3(B,J)]. The colocalization data indicate that replacement of the putative gephyrin binding domain in $\alpha5$ with $\alpha2$GBD, representing a high affinity gephyrin binding site, increased $\alpha5$ synaptic levels, while disruption with the $\alpha4$GBD decreased, but did not eliminate, $\alpha5$ synaptic levels. The remaining association of $\alpha5$ and gephyrin seen here may be due to a gephyrin interaction with an endogenous $\alpha1$ or $\alpha2$ subunit, as previous studies have shown mixed subunit populations occurring *in vivo* (Araujo et al., 1999; del Rio et al., 2001). In summary, these data show that the $\alpha5$ gephyrin binding domain significantly contributes to $\alpha5$ $GABA_AR$ synaptic localization.

Proper Localization of α5-Containing $GABA_A$Rs Is Important for Neuronal Development

As previous genetic and pharmacological studies have implicated a role of $\alpha5$-containing $GABA_ARs$ in proper neuronal development (Curia et al., 2009; Giusi et al., 2009; Fatemi et al., 2010) we examined the effect of $\alpha5$ redistribution on neuronal morphology at DIV 14 and 21. Neurons were transfected with the $\alpha5^{WT}$, $\alpha5^{\alpha4GBD}$, or $\alpha5^{\alpha2GBD}$ constructs at plating, then fixed, permeabilized, and stained with anti-GFP and anti-MAP-2. 3-D reconstructions of confocal z stacks were analyzed by Sholl analysis using ImageJ. At DIV 14, $\alpha5^{\alpha2GBD}$ neurons exhibited a significant decrease in the number of intersections compared to $\alpha5^{WT}$ and $\alpha5^{\alpha4GBD}$ [two-way ANOVA significant effect of transfection $p = 0.0116$; Tukey *post hoc* test $\alpha5^{\alpha2GBD}$ vs. $\alpha5^{WT}$ $p < 0.05$; $\alpha5^{\alpha2GBD}$ vs. $\alpha5^{\alpha4GBD}$ $p < 0.05$; Fig. 4(A,C)]. At DIV 21, we observed a significant decrease in the number of intersections between $\alpha5^{\alpha2GBD}$ neurons compared to $\alpha5^{WT}$ and $\alpha5^{\alpha4GBD}$ [two-way ANOVA significant effect of transfection $p < 0.0001$; Tukey *post hoc* test $\alpha5^{\alpha2GBD}$ vs. $\alpha5^{WT}$ $p < 0.05$; $\alpha5^{\alpha2GBD}$ vs. $\alpha5^{\alpha4GBD}$ $p < 0.0001$ Fig. 4(B,D)], and a significant increase in the number of intersections between $\alpha5^{\alpha4GBD}$ and $\alpha5^{WT}$ [$p < 0.05$ Fig. 4(B,D)]. Further analysis found no significant difference in number of primary, secondary, or tertiary dendrites, or total dendritic length, either at DIV 14 or 21 (Supporting Information Fig. S3). Together, these data indicate that shifting $\alpha5$ localization into the synapse via the $\alpha2$ GBD impairs dendritic

outgrowth, while moving the $\alpha 5$ subunit out of the synapse via the $\alpha 4$ GBD increases dendritic outgrowth.

We also examined the effect of $\alpha 5$ mislocalization on dendritic spine morphology, since alterations in $GABA_AR$ surface levels have been shown to alter spine maturation (Jacob et al., 2009). At DIV 14, while overall spine length and spine density were unchanged (Supporting Information Fig. S3), there was a significant decrease in the number of mushroom spines in $\alpha 5^{\alpha 4GBD}$ neurons compared to $\alpha 5^{WT}$ and $\alpha 5^{\alpha 2GBD}$ [$\alpha 5^{\alpha 4GBD}$ 2.502 ± 0.16 spines/10 μm, $\alpha 5^{WT}$ 4.48 ± 0.46 spines/10 μm, $\alpha 5^{\alpha 2GBD}$ 4.50 ± 0.38 spines/10 μm; $\alpha 5^{\alpha 4GBD}$ vs. $\alpha 5^{WT}$ $p = 0.016$; $\alpha 5^{\alpha 4GBD}$ vs. $\alpha 5^{\alpha 2GBD}$ $p = 0.0083$, Fig. 5(A,C)], which led to a shift in mushroom/filopodia ratio in $\alpha 5^{\alpha 4GBD}$ neurons compared to $\alpha 5^{WT}$ and $\alpha 5^{\alpha 2GBD}$ [$\alpha 5^{\alpha 4GBD}$ 0.73 ± 0.033, $\alpha 5^{WT}$ 1.66 ± 0.24, $\alpha 5^{\alpha 2GBD}$ 2.34 ± 0.38; $\alpha 5^{\alpha 4GBD}$ vs. $\alpha 5^{WT}$ $p = 0.019$; $\alpha 5^{\alpha 4GBD}$ vs. $\alpha 5^{\alpha 2GBD}$ $p = 0.0131$, Fig. 5(A,B)]. At DIV 21, the number of mushroom spines in $\alpha 5^{\alpha 4GBD}$ neurons were still reduced compared to $\alpha 5^{WT}$ [$\alpha 5^{\alpha 4GBD}$ 3.50 ± 0.37, $\alpha 5^{WT}$ 4.90 ± 0.32, $\alpha 5^{\alpha 2GBD}$ 4.46 ± 0.23; $\alpha 5^{\alpha 4GBD}$ vs. $\alpha 5^{WT}$ $p = 0.0461$, Fig. 5(E,G)], again leading to a decrease in the mushroom/filopodia ratio [$\alpha 5^{\alpha 4GBD}$ 1.02 ± 0.14, $\alpha 5^{WT}$ 2.45 ± 0.34, $\alpha 5^{\alpha 2GBD}$ 1.56 ± 0.30; $\alpha 5^{\alpha 4GBD}$ vs. $\alpha 5^{WT}$ $p = 0.018$, Fig. 5(E,F)]. Neither spine density nor lengths were altered at DIV 21 (Supporting Information Fig. S4). The decrease in the mushroom/filopodia ratio suggests that a shift of $\alpha 5$-containing $GABA_ARs$ out of the GABAergic synapse results in a less mature spine, and that this immature phenotype is maintained throughout development.

DISCUSSION

$\alpha 5$ $GABA_ARs$ are emerging as key contributors to learning and memory processes and potential targets for pharmacological modulation in treating cognitive and neurodevelopmental disorders, with clinical trials ongoing in Down syndrome treatment (Rudolph and Mohler, 2014). Extrasynaptically localized $\alpha 5$ $GABA_ARs$ generate tonic inhibition in the hippocampus. However, the function and contribution of synaptically localized $\alpha 5$ $GABA_AR$ to inhibition has largely been overlooked. To resolve this issue, we investigated the localization of the $\alpha 5$ $GABA_AR$ sub-type during development, the mechanism of $\alpha 5$ restriction at synapses, and how its regulated distribution at synaptic versus extrasynaptic sites contributes to the function of $\alpha 5$ in neuronal development. We found that a significant portion of GABAergic post-synaptic compartments contain the $\alpha 5$ $GABA_AR$ subunit, both *in vitro* and *in vivo*. Surface $\alpha 5$ $GABA_ARs$ are significantly colocalized with gephyrin in hippocampal neurons, with $\alpha 5$ synaptic content being maintained at a constant ratio from 7 to 21 DIV. The association between gephyrin and the $\alpha 5$ subunit occurs independent of other α subunits that bind gephyrin and requires Residues 370–385 of the $\alpha 5$ subunit. By exchanging these residues with the corresponding residues of either the high affinity gephyrin binding domain of the $\alpha 2$ subunit or homologous residues from the extrasynaptic $\alpha 4$ subunit that does not interact with gephyrin, we created chimeras that shifted the localization of the $\alpha 5$ subunit without changing $\alpha 5$ total surface levels, thus altering the synaptic/extrasynaptic ratio of $\alpha 5$. Redistribution of the $\alpha 5$ subunit had significant functional consequences, as these chimeras disrupted proper neuronal dendritic morphology and spine maturation.

A role for $\alpha5$ $GABA_AR$ in dendritic development is supported from *in vitro* pharmacological and genetic studies. $\alpha5$-inverse agonist treatment of hippocampal neurons decreased dendritic arborization and reduced glutamate receptor expression (Giusi et al., 2009). Furthermore, neurodevelopmental disorders, such as autism and Fragile X syndrome, have demonstrated alterations in $\alpha5$ subunit levels (Curia et al., 2009; Mendez et al., 2013). However, the precise function of $\alpha5$-containing $GABA_AR$ signaling, particularly of the prevalent synaptic $\alpha5$ $GABA_AR$ population identified here, in dendritic outgrowth is not known. The $\alpha5^{\alpha4GBD}$ and $\alpha5^{\alpha2GBD}$ chimeras provided the opportunity to assess the contributions of $\alpha5$ synaptic and extrasynaptic signaling to neuronal development. Replacing the $\alpha5$ GBD with the $\alpha2$ GBD increased $\alpha5$-containing $GABA_AR$ synaptic localization similar to levels previously reported for the endogenous $\alpha2$ subunit (Tretter et al., 2008). We found that shifting the $\alpha5$ subunit into the synapse reduced the number of dendritic intersections in Sholl analysis compared to wild type at DIV 14 and 21, indicating impaired dendritic growth. Conversely, moving the $\alpha5$ subunit out of the synapse, by way of the $\alpha5^{\alpha4GBD}$ chimera, increased the number of intersections at DIV 21. Previous studies have shown that both tonic and phasic GABAergic inhibition can control Ca^{2+} transients in dendrites (Pan and Lipton, 1995; Kanemoto et al., 2011; Hayama et al., 2013), making this a possible mechanism by which altering the synaptic and extrasynaptic $\alpha5$ levels may influence dendritic outgrowth. While the increased dendritic outgrowth observed at DIV 21 in neurons transfected with the $\alpha5^{\alpha4GBD}$ chimera implies enhanced neuronal maturation, it is also possible that decreasing the ratio of synaptic/extrasynaptic $\alpha5$ $GABA_AR$ signaling reduced dendritic pruning. Few studies have examined the role of $GABA_AR$ in dendrite pruning, although one study found that propofol, an anesthetic agent that binds to $GABA_AR$ β subunits and potentiates $GABA_AR$, results in $GABA_AR$-dependent neurite retraction in primary cortical cultures (Turina et al., 2008). It is also known that local Ca^{2+} transients, both through ion channels and intracellular Ca^{2+} stores, can help to stabilize dendrites, and alterations in these transients may result in dendrite retraction (Wong and Ghosh, 2002; Kanamori et al., 2013). A recent study found that glycogen synthase kinase 3β (GSK3β) can regulate dendrite retraction in hippocampal cultures between DIV 14–18 by regulating $\gamma2$ surface expression (Rui et al., 2013). In neurons expressing $\alpha5^{\alpha4GBD}$, the shift of the $\alpha5$ subunit from the synapse to the extrasynaptic membrane may alter the overall excitatory/inhibitory balance, which in turn alters local Ca^{2+} signaling and affects dendritic pruning.

We also found that altering the ratio of synaptic/extrasynaptic $\alpha5$ $GABA_AR$ affected spine maturation. Shifting the $\alpha5$ subunit out of the synapse resulted in a less mature dendritic spine phenotype. This suggests a specific role for $\alpha5$ synaptic signaling in spine development, as removing extrasynaptic $\alpha5$ via $\alpha5^{\alpha2GBD}$ had no significant effect on spine maturity. As with dendritic morphology, the role of synaptic and extrasynaptic GABAergic signaling has not been fully examined in spine development. GABAergic inhibition, primarily through GABAergic synaptic signaling, can focally suppress Ca^{2+} transients on individual spines (Chiu et al., 2013), and induce spine shrinkage or elimination (Hayama et al., 2013). Furthermore, alterations to other $GABA_AR$ subunits, such as the $\alpha1$ and $\beta3$ subunits, disrupted spine maturation in neurons (Heinen et al., 2003; Jacob et al., 2009). Less mature spines show enhanced Ca^{2+} diffusion between the spine head and dendrite

resulting in lower spine calcium accumulation (Zito et al., 2009), which could also play a role in the changes in dendritic morphology seen here.

A large body of evidence indicates that alpha subunit type plays a direct role in controlling $GABA_AR$ localization via gephyrin interactions (Tretter et al., 2008, 2011; Saiepour et al., 2010; Mukherjee et al., 2011; Wu et al., 2012). It has been hypothesized that gephyrin trimers provide multiple binding sites (Sola et al., 2004; Fritschy et al., 2008; Tretter et al., 2012; Specht et al., 2013), such that two subunits within a $GABA_AR$ may bind the gephyrin scaffold. Our study supports a direct interaction between the $\alpha5$ subunit and gephyrin, which would alter receptor avidity for gephyrin and diffusion within and out of the synapse. This has implications for both $\alpha5/\alpha5$ and mixed alpha subunit $GABA_AR$, which exist *in vivo* (Araujo et al., 1999; del Rio et al., 2001). To assess alpha subunit synaptic localization independent of receptor oligomers, a recent study compared $\alpha5$ or $\alpha2$ subunit intracellular domains fused to a single pass transmembrane domain, revealing reduced colocalization of the $\alpha5$ chimera with gephyrin and a higher diffusion rate (Gerrow and Triller, 2014). This has significant implications for the receptor composition of inhibitory synapses, as a mixed $\alpha2/\alpha5$ receptor would diffuse out of the synapse faster than a homogenous $\alpha2/\alpha2$ receptor, but slower than an $\alpha5/\alpha5$ receptor. This subtle tuning of synaptic GABAergic signaling could contribute to neuronal development by modulating local spine and dendritic Ca^{2+} influx.

In conclusion, we found that a significant portion of GABAergic postsynaptic compartments contain the $\alpha5$ $GABA_AR$ subunit, both *in vitro* and *in vivo*. Shifting the synaptic/extrasynaptic localization of $\alpha5$ $GABA_AR$ through substitution of the $\alpha5$ gephyrin binding domain disrupts proper neuronal development, indicating that an appropriate balance of phasic and tonic $\alpha5$ GABAergic inhibition regulates dendritic outgrowth and spine maturation. Further research is needed to elucidate the precise roles of synaptic and extrasynaptic $\alpha5$ $GABA_AR$ at early stages of neuronal circuit development and later in learning and memory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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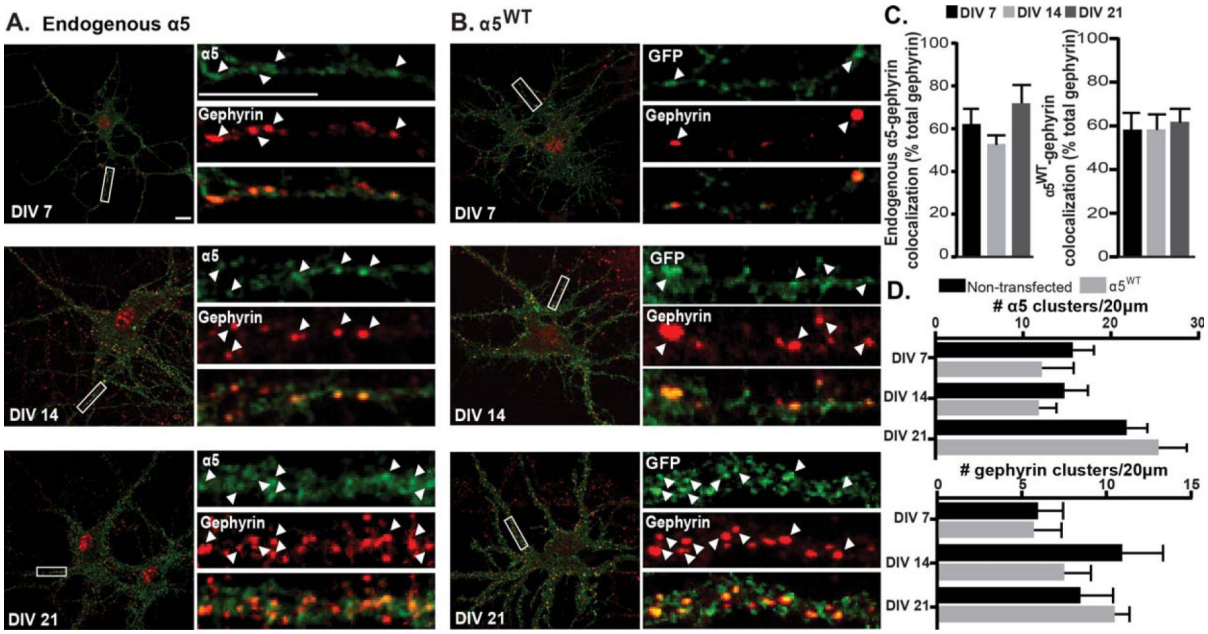

Figure 1.

$GABA_AR$ $\alpha5$ association with gephyrin throughout development in hipppocampal neurons. (A) DIV 7, 14, and 21 neurons were fixed and immunostained for surface $\alpha5$ $GABA_AR$ (anti-$\alpha5$, green, non permeabilized conditions) and gephyrin (red, permeabilized conditions). Colocalized clusters are indicated by arrowheads. Scale bar = 10 μm. (B) Neurons expressing $\alpha5^{WT}$ were fixed and stained for surface tagged $GABA_AR$ (anti-GFP, green, nonpermeabilized conditions) and gephyrin (red). (C) The percentage of gephyrin clusters colocalized with surface clusters of endogenous $\alpha5$ or tagged $\alpha5$ $GABA_AR$ remained constant throughout development. (D) The number of surface $\alpha5$ $GABA_AR$ and gephyrin clusters in 20 μm dendritic segments in nontransfected and transfected cells was compared. No significant difference was found between groups for either $\alpha5$ or gephyrin.

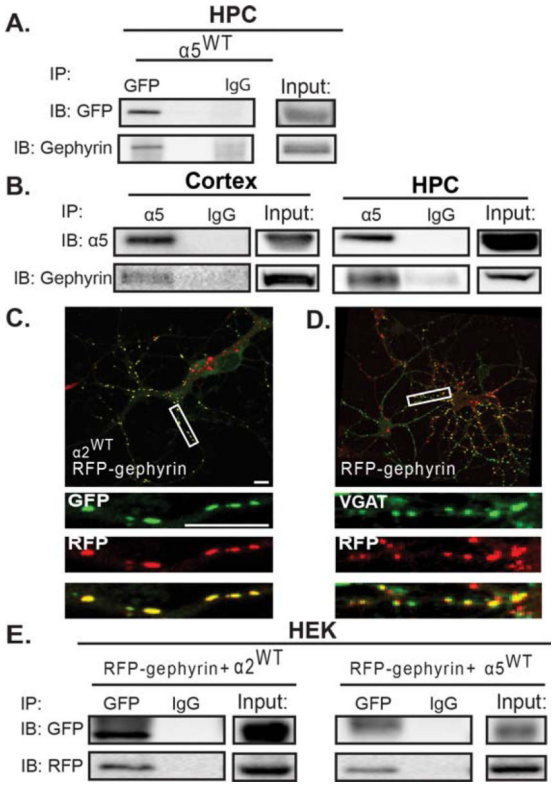

Figure 2.

The $GABA_AR$ $\alpha5$ subunit is able to directly associate with gephyrin. (A) Cultured hippocampal neurons were transfected with $\alpha5^{WT}$ and lysates were immunoprecipitated with anti-GFP or anti-IgG. Precipitates were immunoblotted for GFP and gephyrin. Immunoprecipitation of GFP resulted in coimmunoprecipitation of gephyrin. (B) Cortical and hippocampal (HPC) lysates from adult rat brain were immunoprecipitated with either anti-$\alpha5$ or anti-IgG and immunoblotted for $\alpha5$ and gephyrin. In both cortex and HPC, gephyrin coimmunoprecipitates with $\alpha5$ ($n = 3$ animals). (C) Neurons were transfected with RFP-gephyrin and $\alpha2^{WT}$, then fixed, stained under nonpermeabilized conditions with anti-GFP, then permeabilized and stained with anti-RFP. (D) Neurons were transfected with RFP-gephyrin, fixed, permeabilized, and stained for RFP and VGAT. (E) HEK cells were transfected with $\alpha5^{WT}$, $\beta3$, and RFP-gephyrin. Lysates were then immunoprecipitated for GFP and immunoblotted for RFP and GFP. GFP immunoprecipitation resulted in coimmunoprecipitation of RFP-gephyrin. HEK cells transfected with $\alpha2^{WT}$, $\beta3$, and RFP-gephyrin were used as a positive control.

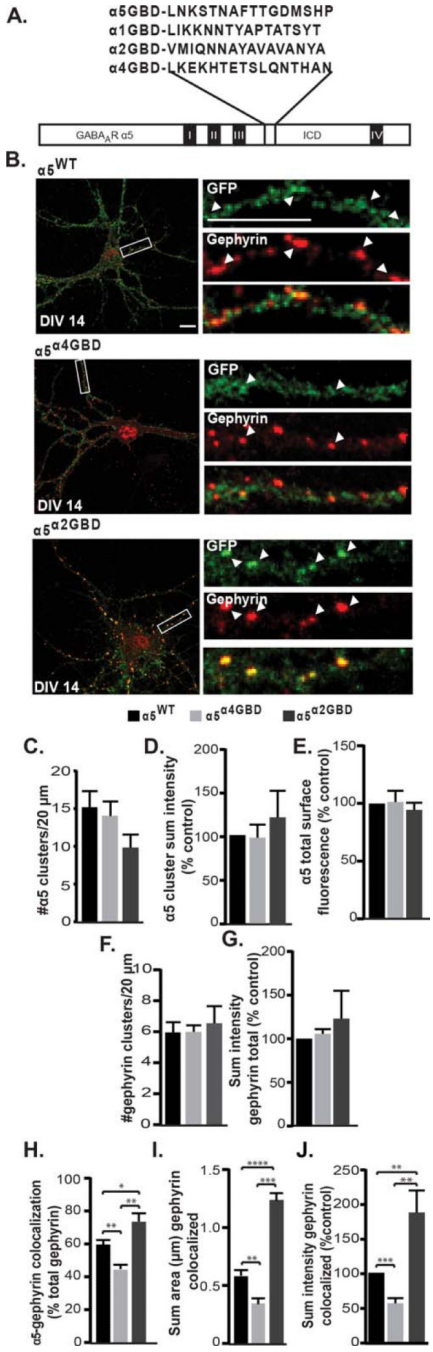

Figure 3.

Disruption of the gephyrin binding domain in the α5 subunit decreases its association with gephyrin. (A) Alignment of homologous regions of the gephyrin binding domain (GBD) of different alpha subunits, and construction of the $\alpha5^{\alpha4GBD}$ and $\alpha5^{\alpha2GBD}$ chimeras. (B) DIV 14 neurons expressing the $\alpha5^{WT}$, $\alpha5^{\alpha4GBD}$, or $\alpha5^{\alpha2GBD}$ constructs were fixed and stained for surface GABA$_A$R (anti-GFP, green) and gephyrin (red). (C–E) In transfected neurons, there was no significant difference in the total number of α5 clusters per 20 μm, the total sum intensity of α5 clusters, or the total α5 surface fluorescence. (F and G) There was no

significant difference in the total number of gephyrin clusters per 20 μm or the overall sum intensity of gephyrin between groups. (H) Swapping the α5 GBD for the α4 GBD significantly decreased colocalization of the α5 subunit with gephyrin, while exchanging the α5 GBD for the α2 GBD significantly increased gephyrin colocalization (*$p < 0.05$, **$p < 0.01$). (I and J) The sum area and sum intensity of gephyrin clusters colocalized with tagged $GABA_AR$ was significantly decreased in neurons transfected with the $\alpha5^{\alpha4GBD}$ compared to control, while neurons transfected with $\alpha5^{\alpha2GBD}$ showed a significant increase in sum area and sum intensity of colocalized gephyrin clusters (**$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

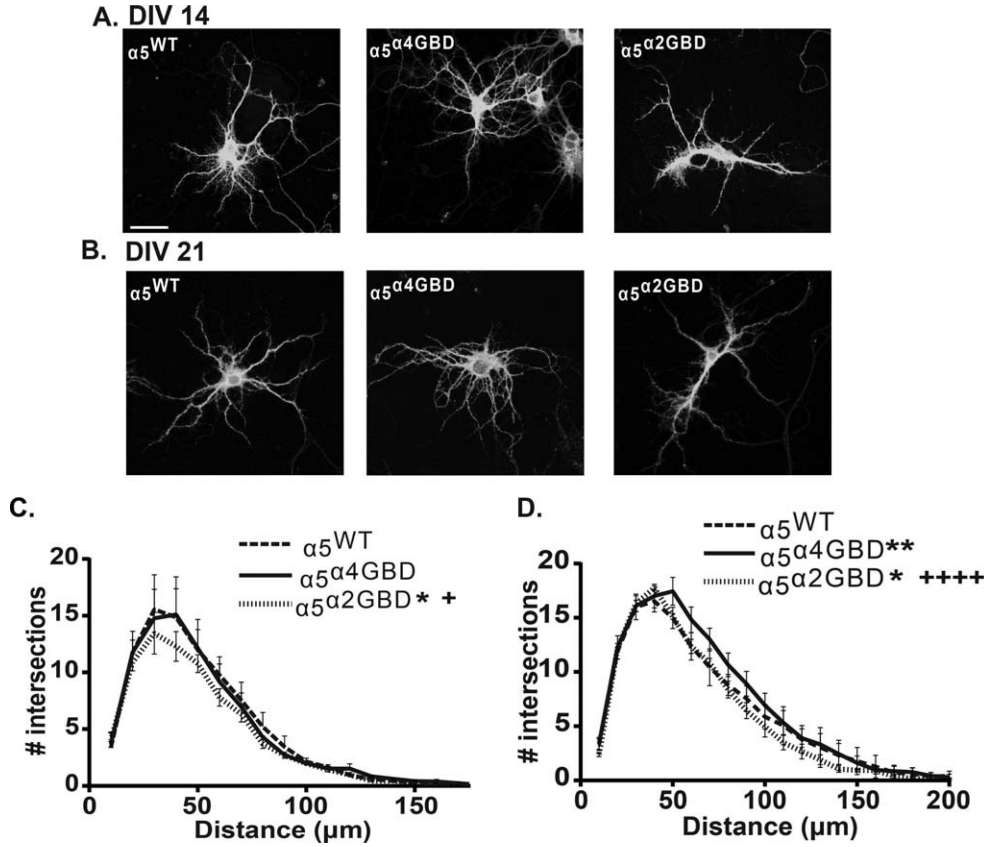

Figure 4.

$\alpha 5$ localization is important in controlling dendritic outgrowth. Sholl analysis was performed on hippocampal cultures transfected with the $\alpha 5^{WT}$, $\alpha 5^{\alpha 4GBD}$, or $\alpha 5^{\alpha 2GBD}$, then fixed and stained under permeabilized conditions with anti-GFP and anti-MAP-2 antibodies. Confocal z-series were acquired with a 40× objective, and 3D constructions were used to analyze dendritic morphology. (A and C) At DIV 14, neurons transfected with $\alpha 5^{\alpha 2GBD}$ exhibited impaired dendritic growth compared to those transfected with $\alpha 5^{WT}$ or $\alpha 5^{\alpha 4GBD}$ ($^{*}p < 0.05$ compared to $\alpha 5^{WT}$, $^{+}p < 0.05$ compared to $\alpha 5^{\alpha 4GBD}$; number of dendrites examined: $\alpha 5^{WT}$ 1054, $\alpha 5^{\alpha 4GBD}$ 1076, $\alpha 5^{\alpha 2GBD}$ 1020). (B and D) At DIV 21, neurons transfected with $\alpha 5^{\alpha 4GBD}$ showed more dendritic intersections than either $\alpha 5^{WT}$ or $\alpha 5^{\alpha 2GBD}$, while neurons expressing $\alpha 5^{\alpha 2GBD}$ showed fewer dendritic intersections ($^{*}p <$ 0.05 compared to $\alpha 5^{WT}$, ** $p < 0.01$ compared to $\alpha 5^{WT}$, $^{++++}p < 0.0001$ compared to $\alpha 5^{\alpha 4GBD}$; number of dendrites examined: $\alpha 5^{WT}$ 1126, $\alpha 5^{\alpha 4GBD}$ 1213, $\alpha 5^{\alpha 2GBD}$ 1269).

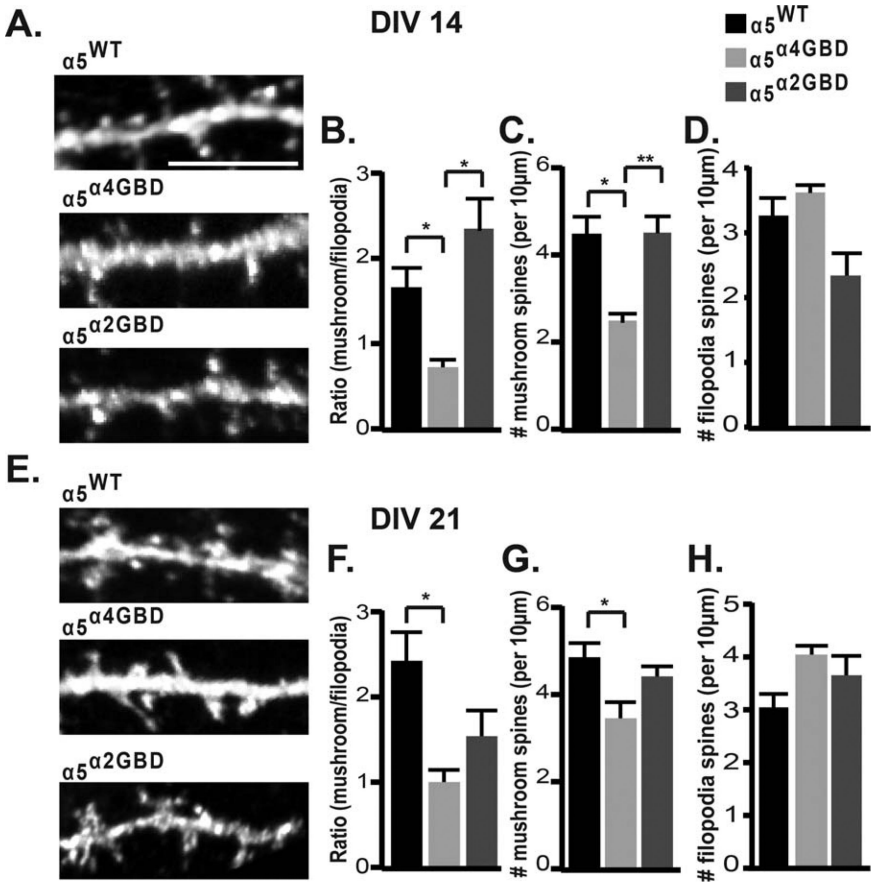

Figure 5.

Altered ratio of synaptic/extrasynaptic $\alpha 5$ $GABA_AR$ disrupts spine maturation. Neurons were transfected with $\alpha 5^{WT}$, $\alpha 5^{\alpha 4GBD}$, or $\alpha 5^{\alpha 2GBD}$, then fixed and stained under permeabilized conditions with anti-GFP and anti-MAP-2 antibodies. High magnification confocal z-series through dendritic regions were obtained, and 3D reconstructions were used to analyze spine length, density, and morphology. 3D reconstructions of confocal images from neurons expressing $\alpha 5^{WT}$, $\alpha 5^{\alpha 4GBD}$, or $\alpha 5^{\alpha 2GBD}$ at DIV 14 (A) and DIV 21 (E). (B) At DIV 14, neurons expressing $\alpha 5^{\alpha 4GBD}$ exhibited a significantly lower mushroom/filopodia spine ratio compared to either $\alpha 5^{WT}$ or $\alpha 5^{\alpha 2GBD}$ (for B–H, $*p < 0.05$, $**p < 0.01$; number of spines examined: $\alpha 5^{WT}$ 394, $\alpha 5^{\alpha 4GBD}$ 311, $\alpha 5^{\alpha 2GBD}$ 358). (C) Neurons expressing $\alpha 5^{\alpha 4GBD}$ exhibited significantly fewer mushroom spines per 10 μm compared to either $\alpha 5^{WT}$ or $\alpha 5^{\alpha 2GBD}$. (F) At DIV 21, neurons expressing $\alpha 5^{\alpha 4GBD}$ exhibited a significantly lower mushroom/filopodia spine ratio compared to $\alpha 5^{WT}$ (number of spines examined: $\alpha 5^{WT}$ 474, $\alpha 5^{\alpha 4GBD}$ 453, $\alpha 5^{\alpha 2GBD}$ 455). (G) Neurons expressing $\alpha 5^{\alpha 4GBD}$ exhibited significantly fewer mushroom spines per 10 μm compared to $\alpha 5^{WT}$. There was no significant difference between the number of filopodia spines per 10 μm between constructs at DIV 14 (D) or at DIV 21 (H).