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Postnatal Down-Regulation of the $GABA_A$ Receptor γ_2 Subunit in Neocortical NG2 Cells Accompanies Synaptic-to-Extrasynaptic Switch in the GABAergic Transmission Mode

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NG2 cells, a main pool of glial progenitors, express γ-aminobutyric acid A ($GABA_A$) receptors ($GABA_A$Rs), the functional and molecular properties of which are largely unknown. We recently reported that transmission between GABAergic interneurons and NG2 cells drastically changes during development of the somatosensory cortex, switching from synaptic to extrasynaptic communication. Since synaptic and extrasynaptic $GABA_A$Rs of neurons differ in their subunit composition, we hypothesize that $GABA_A$Rs of NG2 cells undergo molecular changes during cortical development accompanying the switch of transmission modes. Single-cell RT–PCR and the effects of zolpidem and α5IA on evoked GABAergic currents reveal the predominance of functional α1- and α5-containing $GABA_A$Rs at interneuron–NG2 cell synapses in the second postnatal week, while the α5 expression declines later in development when responses are exclusively extrasynaptic. Importantly, pharmacological and molecular analyses demonstrate that γ2, a subunit contributing to the clustering of $GABA_A$Rs at postsynaptic sites in neurons, is down-regulated in NG2 cells in a cell type-specific manner in concomitance with the decline of synaptic activity and the switch of transmission mode. In keeping with the synaptic nature of γ2 in neurons, the down-regulation of this subunit is an important molecular hallmark of the change of transmission modes between interneurons and NG2 cells during development.

Keywords: GABA spillover, $GABA_A$ receptor γ2 subunit, oligodendrocyte precursor cell, single-cell RT–PCR, synaptic transmission

Introduction

γ-Aminobutyric acid A ($GABA_A$) receptors ($GABA_A$Rs) form heteropentameric complexes from a family of 19 subunits, such as α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3. In the brain, they are generally composed of an association of αβγ subunits with a 2α–2β–1γ stoichiometry. The diversity in subunit composition results in distinct agonist affinities and channel kinetics. The mechanisms of regulation and targeting of $GABA_A$Rs at the membrane are also differently influenced by specific subunits (Farrant and Nusser 2005). While the γ2 subunit is an important component of synaptic $GABA_A$Rs and an interacting partner of the scaffolding protein gephyrin (Essrich et al. 1998; Schweizer et al. 2003), α4, α5, α6, and δ subunits are mainly considered as extrasynaptic (Farrant and Nusser 2005). As a physiological consequence of this remarkable molecular heterogeneity and subunit specificity, synaptic and extrasynaptic $GABA_A$Rs with different subunit composition distinctly mediate phasic and tonic inhibition in neurons (Farrant and Nusser 2005).

Major types of glia and glial progenitors also express functional $GABA_A$Rs, but the molecular rules that govern the function of these receptors in non-neuronal cells are poorly understood (Velez-Fort et al. 2012). Interestingly, oligodendrocyte precursor cells expressing the proteoglycan NG2 (NG2 cells), a major pool of progenitors in the postnatal brain (Nishiyama et al. 2009), express functional $GABA_A$Rs that are activated directly by GABA release at bona fide synapses with neurons (Lin and Bergles 2004; Jabs et al. 2005; Gallo et al. 2008). In the juvenile hippocampus, postsynaptic receptors of NG2 cells are mainly composed of α1, α2, β3, γ1, and γ2 subunits (Passlick et al. 2013). We recently demonstrated that GABAergic synaptic activity in NG2 cells of the somatosensory cortex is transient during postnatal development, with a higher frequency of synaptic currents found during the second postnatal week and disappearing at later stages (Vélez-Fort et al. 2010). In the more mature brain, a local mode of extrasynaptic communication that does not rely on the presence of synaptic input, but involves a form of GABA spillover, is established after the loss of synapses (Vélez-Fort et al. 2010). This postnatal switch from synaptic to extrasynaptic transmission suggests that molecular and cellular mechanisms underlying interneuron–NG2 cell signaling are developmentally regulated. In particular, $GABA_A$Rs of NG2 cells may undergo molecular modifications in concomitance with the switch of transmission. However, the molecular and functional properties of native $GABA_A$Rs in neocortical NG2 cells are unknown, and contradictory results concerning the expression of the γ2 subunit have been reported in cultured oligodendrocyte precursor cells (Von Blankenfeld et al. 1991; Bronstein et al. 1998; Williamson et al. 1998).

Here, we investigated whether the properties of $GABA_A$Rs in NG2 cells of the barrel cortex change during postnatal development. By combining patch-clamp recordings, pharmacological analysis, and single-cell RT–PCR of $GABA_A$R subunits, we demonstrate that subunit expression of $GABA_A$Rs in NG2 cells undergoes important molecular modifications during the first postnatal month. Notably, we demonstrated a down-regulation of the γ2 subunit in NG2 cells that occurs in concomitance with the reduction of synaptic connectivity and the switch of transmission modes during the second postnatal week. Our results suggest that this molecular change of $GABA_A$Rs in NG2 cells

impacts the GABAergic mode of transmission to these progenitor cells during cortical development.

Materials and Methods

Slice Preparation and Electrophysiology

All experiments followed European Union and institutional guidelines for the care and use of laboratory animals. Acute parasagittal slices (300 µm) of the barrel cortex were performed in NG2-DsRed BAC transgenic mice as previously described (Vélez-Fort et al. 2010). Patch-clamp recordings of DsRed$^+$ NG2 cells were performed from postnatal days (PN) 7 to PN11 (PN7–11), PN12–14, and PN21–29. Recordings were performed at 30–32 °C using an extracellular solution containing (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 20 glucose, 5 pyruvate, 2 $CaCl_2$, and 1 $MgCl_2$ (95% O_2 and 5% CO_2). The intracellular solution was a CsCl-based solution containing (in mM): 130 CsCl, 10 4-aminopyridine, 5 tetraethylammonium chloride, 5 EGTA, 0.5 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 2 Na_2ATP, 0.2 Na-GTP, and 10 Na_2-phosphocreatine (pH ≈ 7.3, 296 mOsm). For single-cell RT–PCR analysis, we used a KCl-based intracellular solution containing (in mM): 130 KCl, 5 BAPTA, 2 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, and 3 Na_2ATP (pH 7.28).

Extracellular stimulations were obtained using a monopolar electrode (glass pipette) placed in layer V of the barrel cortex, while recording NG2 cells held at −70 mV in the same layer. The rate of stimulation was 0.067 Hz at PN7–14 and reduced to 0.04 Hz at PN21–29 to avoid rundown of GABAergic extrasynaptic responses (3–40 V; 100 µs stimulations; Iso-Stim 01D, npi electronic GmbH, Tamm, Germany). To isolate $GABA_A$R currents, recordings were performed in 50 µM D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5), 10 µM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoxaline-7-sulfonamide (NBQX), and 5 µM CGP 55845 (or CGP 46381), antagonists for NMDA, AMPA/kainate, and $GABA_B$ receptors, respectively. In the majority of experiments, we also added 100 µM Ba^{2+} to the perfusate to block Kir4.1 channels, particularly in the fourth postnatal week, and to increase the membrane resistance of recorded cells, as previously reported (Maldonado et al. 2011, 2013). In these conditions, the membrane resistance of NG2 cells was 3.93 ± 6.12 (n = 31), 1.55 ± 1.33 (n = 16), and 1.55 ± 1.17 GΩ (n = 30) at PN7–11, PN12–14, and PN21–29, respectively (P < 0.05). Recordings were made without series resistance (R_s) compensation; R_s was monitored during recordings and cells showing a change of >30% in R_s were discarded. Whole-cell recordings were obtained using Multiclamp 700B, filtered at 2–4 kHz, and digitized at 20 kHz. Digitized data were analyzed off-line using pClamp 10.1 (Molecular Devices) and Igor Pro 6.0. Miniature GABAergic currents were analyzed by using SpAcAn in Igor Pro (Dugue et al. 2009; http://www.spacan.net/).

The amplitude of evoked currents was measured on average traces in control conditions and after 3 min of drug application. The rise time ($t_{20–80\%}$) was measured only in cells where the onset of evoked responses was not masked by the stimulation artifact. The rectification index from current–voltage (I–V) curves was calculated as follows: $RI = [I_{35}/(35\text{ mV} - E_{rev})]/[I_{-70}/(-70\text{ mV} - E_{rev})]$, where I_{35} and I_{-70} correspond to the current at 35 and −70 mV, respectively, and E_{rev} corresponds to the experimental reversal potential. Currents were normalized with respect to the current at −70 mV. The paired-pulse ratio of evoked currents was estimated by averaging 15–75 sweeps and by dividing the current amplitude of the second pulse by that of the first pulse. Miniature evoked GABAergic synaptic currents were detected with a detection threshold of 3 times the standard deviation (SD) of the current noise. The frequency of evoked miniature events obtained by replacing 2 mM Ca^{2+} by 5 mM Sr^{2+} was calculated during 1.5 s after stimulation and compared with the frequency of events prior to the stimulation during the same duration. The frequency of evoked miniature events was significantly higher than that measured before stimulation (0.15 ± 0.04 and 0.55 ± 0.12 Hz before and after the stimulation, n = 16; P < 0.05). The existence of a tonic current was tested for measuring the holding current before and during drug application and by quantifying a noise increase of the trace as a variation of the SD (ΔSD). To estimate the Na^+ current density of NG2 cells, the amplitude of the Na^+ current was measured at −20 mV after leak subtraction and divided by the cell capacitance.

Single-Cell RT–PCR for $GABA_A$R Subunits

After recording, the cytoplasm of individual cells was harvested under microscopic control (Seifert et al. 2009). For single-strand cDNA synthesis, 5.5 µL of reaction mix was added to the tubes (final volume of 10 µL) containing first-strand buffer, 10 mM dithiothreitole, deoxyribonucleotide triphosphates (dNTPs, final concentration 4 × 250 µM; Applied Biosystems, Darmstadt, Germany), random hexanucleotide primer (50 µM; Roche, Mannheim, Germany), 20 U RNasin (Promega, Mannheim, Germany), and 100 U Superscript III reverse transcriptase (Invitrogen, Darmstadt, Germany). RT was performed at 37 °C (1 h). The multiplex 2-round single-cell PCRs were performed with primers for $GABA_A$R subunits and the NG2 cell marker PDGFRα. The first PCR was performed after adding PCR buffer, $MgCl_2$ (2.5 mM), primers (200 nM each; PDGFRα 100 nM each), and Taq polymerase (3.5 U; Invitrogen) to the RT product (final volume of 50 µL). Thirty-five cycles were performed (denaturation at 94 °C, 25 s; annealing at 51 °C, 2 min for the first 5 cycles, and 45 s for the remaining cycles; extension at 72 °C, 25 s; final elongation at 72 °C, 7 min). An aliquot (2 µL) of the PCR product was used as a template for the second PCR (35 cycles; annealing at 54 °C, first 5 cycles: 2 min, remaining cycles: 45 s) with nested, subunit-specific primers. The list of primers was previously reported in Passlick et al. (2013), except for the δ subunit for which we used: 5′ TGGCGCCAGGGCAATGAATG 3′ and 5′-GTGGAGGTGATGCGGAT GCTGTAT 3′ for the first PCR, and 5′ TATGCCCGAAACTTCCGACCAG 3′ and 5′-AAAATCACCCCATCAGGCTGTAGG 3′ for the second nested PCR. The α6 subunit was not tested, since this subunit is virtually absent in the neocortex (Laurie et al. 1992). The conditions were the same as described for the first round, but dNTPs (4 × 50 µM) and Platinum Taq polymerase (2.5 U; Invitrogen) were added. To discriminate between the expression of β2 and β3 subunits, the second PCR (β2/3) was repeated. The PCR product was purified (MinElute PCR Purification Kit; Qiagen, Hilden, Germany), and dissolved in 30 µL of H_2O. Seven microliters of the PCR product were incubated in restriction enzyme (10 U, 6 h, 37 °C). The PCR products for β2/3 subunits were digested by *Pst*I (specific to β2) and *Ban*I (cleaving specifically β3). *Pst*I cut the PCR product (307 bp) into 169 and 138 bp fragments, whereas *Ban*I produced fragments of 202 and 105 bp length. Products were identified by gel electrophoresis using a molecular weight marker (Low Molecular Weight DNA Ladder; New England Biolabs, Frankfurt, Germany; 50 bp ladder, Invitrogen). Specificity of primers was tested with 2 ng of total RNA prepared from the freshly isolated mouse brain. Subsequent gel analysis did not detect unspecific products. The primers for the targets were located on different exons to prevent amplification of genomic DNA. Omission of the RT enzyme and substitution of template by bath solution served as negative controls for RT and PCR amplification.

Statistics

Data are expressed as mean ± SEM. The nonparametric Mann–Whitney U-test for independent samples was used to determine statistical differences between data obtained at different postnatal stages. The Wilcoxon signed-rank test for related samples was used when comparisons within single cells were required (GraphPad Prism Software, version 5.00). The degree of linear relationship between 2 variables was measured with the Pearson product–moment correlation coefficient (R designates the correlation coefficient).

Drugs

D-AP5 and NBQX were purchased from Abcam Biochemicals (Cambridge, UK). CGP 55845, CGP 46381, diazepam, and zolpidem were purchased from Tocris Cookson (Bristol, UK). Furosemide and α5IA were purchased from Sigma-Aldrich (St Louis, USA).

Results

Developmental Regulation of GABA$_A$R Subunit mRNA Expression in Cortical NG2 Cells

Synaptic and extrasynaptic GABA$_A$Rs in neurons are assembled from different subunits and have distinct biophysical properties (Farrant and Nusser 2005). We thus hypothesized that GABA$_A$Rs of NG2 cells undergo molecular modifications in concomitance with the switch of transmission mode from synaptic to extrasynaptic that occurs during the first postnatal month in the somatosensory cortex (Vélez-Fort et al. 2010). To get a picture of the repertoire of GABA$_A$R subunits expressed in NG2 cells during postnatal development, we compared the expression pattern of 12 genes by single-cell RT–PCR between PN7–11 and PN21–29, 2 developmental stages corresponding, respectively, to the period of the maximal and minimal frequency of spontaneous synaptic activity in cortical NG2 cells (Fig. 1; see Vélez-Fort et al. 2010 for synaptic current frequency). These genes correspond to the main GABA$_A$R subunits expressed in the neocortex (Laurie et al. 1992; Golshani et al. 1997). Whole-cell recordings of individual NG2 cells were performed in layer V of acute slices of the barrel cortex from NG2-DsRed transgenic mice and subjected to functional characterization; the cytoplasm of these cells was harvested into the recording pipette for single-cell RT–PCR analysis of the transcripts. After RT, we performed a 2-round PCR using primer sets for α, β, and γ subunits in the first round, together with primers for the NG2 cell-specific marker PDGFRα. For the second PCR round, subunit-specific primers were used (Passlick et al. 2013). The presence of mRNA for PDGFRα served as a positive control (Fig. 1*a–d*).

At PN7–11, many of the cortical NG2 cells expressed α1 (62%), α2 (56%), and α5 (44%), whereas α3 and α4 were less abundant (25 and 19%, $n = 16$; Fig. 1*a*,*e*). At PN21–29, we observed a drop in the expression frequency of α2 (37%) and α5 (16%) and an enhanced expression of α3 (52%) and α4 (47%; $n = 19$; Fig. 1*b*,*e*). An age-dependent decline was observed for β1 (from 21%, $n = 19$ to 7%, $n = 14$), whereas β2 and β3 expression were high and age-independent (Fig. 1*f*). All γ subunits were detected in NG2 cells with γ3 being most frequently expressed at PN7–11 (67%; $n = 21$) and at PN21–29 (75%; $n = 20$; Fig. 1*g*). Interestingly, we observed a pronounced, age-dependent drop in the abundance of γ2, a main constituent of synaptic GABA$_A$Rs in neurons, and an interacting partner of the scaffolding protein gephyrin (43% at PN7–11, virtually absent at PN21–29, $n = 21$ and 20, respectively; Fig. 1*c*,*d*,*g*; Essrich et al. 1998). The δ subunit, one major extrasynaptic subunit in some neurons but poorly expressed in the neocortex (Wisden et al. 1992; Farrant and Nusser 2005), was absent at PN7–11 and only rarely detected in older NG2 cells (18%, $n = 11$; Fig. 1*g*).

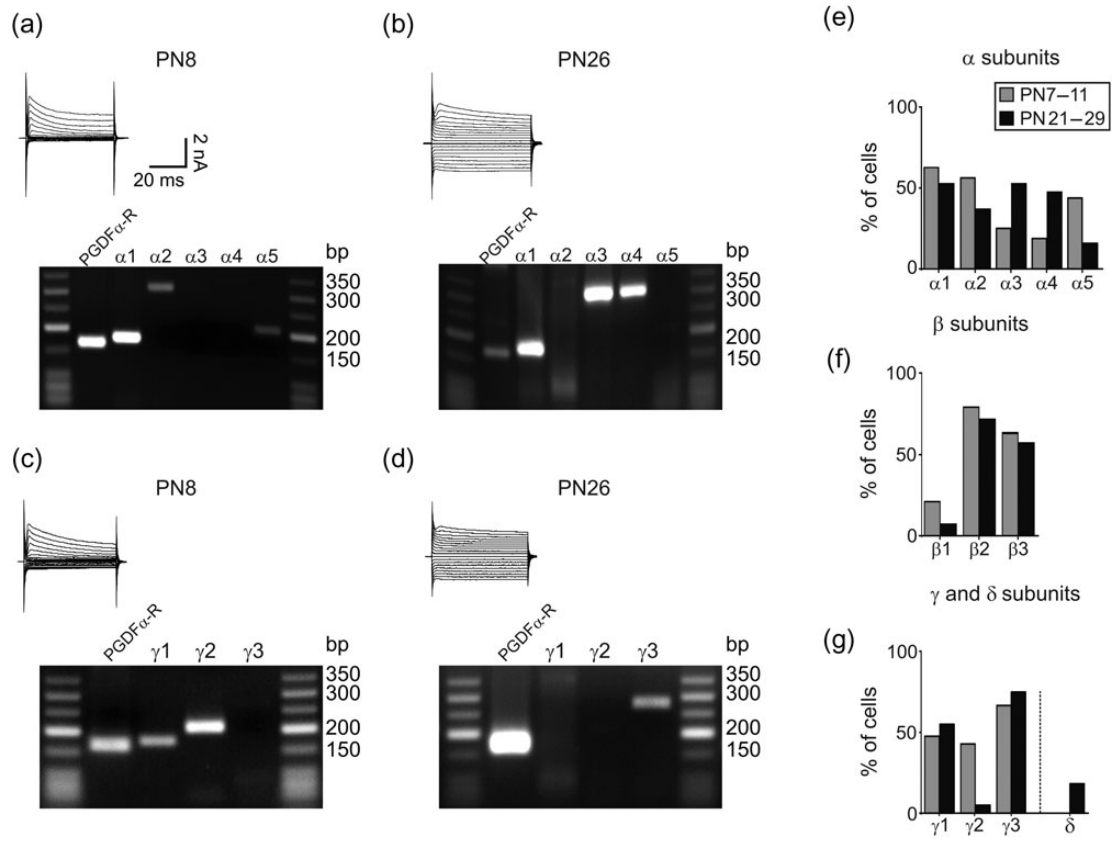

Figure 1. Molecular diversity of GABA$_A$R subunit expression in NG2 cells at PN7–11 and PN21–29. (*a–d*) The co-expression of α (*a* and *b*), β, γ (*c* and *d*), and δ subunit mRNAs were investigated in recorded NG2 cells by single-cell RT–PCR at PN7–11 and PN21–29. Currents were induced in cells held at −70 mV by voltage steps from +20 to −160 mV (10 mV increments; upper-left panels). After recording, the cell content was harvested, and RT–PCR was performed. The gels display co-expression of transcripts for the cell type-specific marker PDGFRα together with α-subunits (*a* and *b*) or γ-subunits (*c* and *d*) of GABA$_A$Rs. The expected product lengths were 167 bp for PDGFRα, 180 bp for α1, 306 bp for α2, 306 bp for α3, 308 bp for α4, 208 bp for α5, 180 bp for γ1, 211 bp for γ2, and 272 bp for γ3. (*e–g*) Summary of GABA$_A$R subunit expression in single NG2 cells observed at PN7–11 and PN21–29. The number of tested cells is 16, 19, 21, and 19 for α, β, γ, and δ subunits at PN7–11, respectively, and 19, 14, 20, and 11 for α, β, γ, and δ subunits at PN21–29, respectively.

Molecular analyses showed a large heterogeneity and complex developmental regulation of α, β, and γ subunit mRNA expression in cortical NG2 cells. Among postnatally regulated $GABA_AR$ subunits in NG2 cells, 3 subunits are relevant in terms of their synaptic or extrasynaptic location. The α4 and α5 subunits are considered as mainly extrasynaptic, whereas γ2 is a main subunit found at synapses in the brain (Farrant and Nusser 2005). On the basis of these initial findings, we tested thereafter whether changes of mRNA expression patterns for these 3 subunits resulted in functional changes associated with the switch of GABAergic transmission modes between interneurons and NG2 cells.

Postnatal Down-Regulation of the α5 Subunit of $GABA_A$Rs in Cortical NG2 Cells

The electrophysiological and pharmacological properties of $GABA_AR$-mediated currents evoked in NG2 cells were examined in layer V of acute barrel cortex slices of NG2-DsRed transgenic mice. Low-frequency stimulations were applied locally with an extracellular electrode placed in the same layer (0.04–0.067 Hz), while NG2 cells were recorded in voltage-clamp at −70 mV with a CsCl-based intracellular solution containing TEA and 4AP, and in the presence of D-AP5, NBQX, CGP 46381, and Ba^{2+} in the extracellular solution. These conditions were used to isolate $GABA_AR$-mediated responses and to increase the input resistance of the recorded cells (see Materials and Methods; Maldonado et al. 2011, 2013). Stimulation of neuronal fibers easily elicited GABAergic currents in NG2 cells at PN7–11 and PN21–29 [Fig. 2; mean amplitude at −70 mV: −44.7 ± 4.9 pA ($n = 40$) and −54.6 ± 6.3 pA ($n = 42$), respectively; $P > 0.05$]. However, as we previously reported, evoked GABAergic currents had slower rise times during the fourth postnatal week compared with younger mice as a consequence of the switch of transmission mode from synaptic to extrasynaptic [Fig. 2*a*,*b*, insets; $t_{20–80\%} = 2.42 \pm 0.31$ ms ($n = 18$) and $t_{20–80\%} = 4.38 \pm 0.31$ ms ($n = 33$) for PN7–11 and PN21–29, respectively; $P < 0.001$; see also Vélez-Fort et al. 2010]. In addition, this change of current kinetics was accompanied by a modification of *I–V* relationships of evoked responses analyzed between −70 and +35 mV (Fig. 2*a*,*b*). Indeed, the *I–V* curve of evoked responses showed a weak inward rectification at PN7–11, whereas it showed an outward rectification at PN21–29 [Fig. 2*c*; rectification index: 0.75 ± 0.09 ($n = 7$) and 1.22 ± 0.16 ($n = 6$), respectively; $P < 0.01$; see Materials and Methods]. This change in the *I–V* curve suggests developmental modifications in the functional properties of $GABA_ARs$ activated in NG2 cells by a synchronous GABA release from neurons. These results, however, did not exclude the existence of extrasynaptic receptors located at distant sites from synapses and activated by ambient GABA rather than by neuronal stimulation. To evaluate the presence of these distant extrasynaptic receptors during development, we bath applied GABA (5 µM) followed by the competitive antagonist of $GABA_ARs$ SR95531 (50 µM; see Sebe et al. 2010 for neurons). Bath application of GABA induces a tiny tonic current in NG2 cells at PN7–11 compared with large currents in neurons (Supplementary Fig. 1). Moreover, while a tonic current persisted in neurons at PN21–29 (Supplementary Fig. 1*b*), it was virtually absent in NG2 cells at this later stage (Supplementary Fig. 1*d*–*e*). Therefore, we concluded that most functional $GABA_ARs$ of NG2 cells are located at synaptic sites or at extrasynaptic locations close to active zones throughout development.

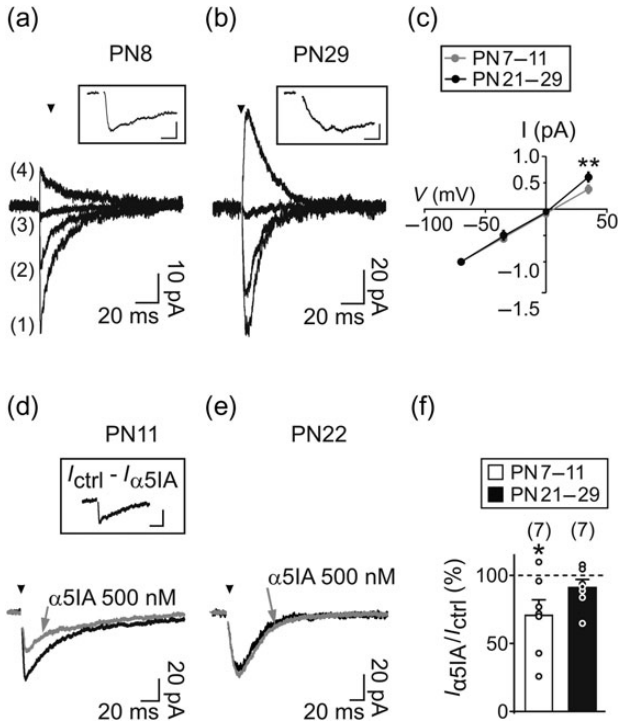

Figure 2. Effect of α5IA on evoked $GABA_AR$-mediated currents of cortical NG2 cells. (*a* and *b*). Evoked $GABA_AR$-mediated currents elicited by stimulation of neuronal fibers in 2 NG2 cells at PN8 (*a*) and PN29 (*b*) at holding potentials of −70 mV (1), −35 mV (2), 0 mV (3), and +35 mV (4). Note the fast rise time of currents elicited at a holding potential of −70 mV in (*a*, inset) compared with (*b*, inset). Inset scale bars: 5 ms, 20 pA (*a*); 5 ms, 40 pA (*b*). (*c*) *I–V* relationships of normalized (−70 mV) evoked currents at both developmental stages. Note the outward rectification of the *I–V* curve of cells at PN21–29. $^{**}P < 0.01$ for currents at +35 mV ($n = 7$ and $n = 6$ at PN7–11 and PN21–29, respectively). (*d* and *e*) Effect of 500 nM α5IA (gray traces) on evoked $GABA_AR$-mediated currents elicited in 2 NG2 cells held at −70 mV at PN11 (*d*) and PN22 (*e*). The inset illustrates the α5IA-sensitive current ($I_{ctrl-\alpha5IA}$) obtained after subtracting the resistant current from the control. Inset scale bar: 20 pA, 20 ms. For all traces, stimulus artifacts were blanked for visibility and the time of stimulation is indicated with a black arrowhead. (*f*) Histogram comparing the effect of 500 nM α5IA in NG2 cells recorded at PN7–11 and PN21–29. $^{*}P < 0.05$.

Transcripts for α4 strongly increase in NG2 cells from PN7–11 to PN21–29 and thus, could constitute a molecular component of extrasynaptic $GABA_ARs$ at a later developmental stage (Fig. 1*e*). To test for the presence of α4-containing $GABA_AR$ in evoked responses of older mice, we used furosemide that inhibits these receptors with relatively high affinity (Wafford et al. 1996; Sun et al. 2007). Bath application of this molecule did not inhibit evoked GABAergic currents of NG2 cells at PN21–29, even at a high concentration of 500 µM (5 times higher than previously used in slices; Sun et al. 2007). These results preclude the presence of functional α4-containing $GABA_ARs$ in older mice, when GABAergic transmission in NG2 cells relies on local GABA spillover [9 ± 5% reduction vs. control ($n = 7$), $P > 0.05$; see Discussion]. It is noteworthy that no tonic current was revealed during the application of the drug [mean current: −1.42 ± 0.62 pA at PN21–29 ($n = 7$), $P > 0.05$].

Contrary to the expression profile of α4, α5 transcript levels are higher in NG2 cells at PN7–11 and decrease at PN21–29 (Fig. 1*e*). We thus tested for the functional expression of α5 in

evoked currents in young and older mice by using its selective inverse agonist α5IA (Dawson et al. 2006; Ali and Thomson 2008). Although the effect of 500 nM α5IA was variable from cell to cell with 2 of 7 insensitive cells at PN7–11 (Fig. 2*f*), bath application of this drug induced on average a significant current reduction at this age (Fig. 2*d*,*f*). On the contrary, current amplitudes were on average not significantly affected by this compound at PN21–29 (Fig. 2*e*,*f*). Thus, as predicted by single-cell RT–PCR analyses, pharmacological experiments showed heterogeneity in α5 subunit expression and, more importantly, an age-dependent down-regulation of functional α5-containing $GABA_A$Rs in NG2 cells. Although this subunit is mainly considered as extrasynaptic, it has also been described at neuronal synapses (Serwanski et al. 2006; Ali and Thomson 2008) and thought to be present at postsynaptic $GABA_A$Rs of hippocampal NG2 cells (Lin and Bergles 2004). To assess whether evoked α5IA-sensitive responses were mediated by synaptic or extrasynaptic receptors in NG2 cells of young mice, we compared the rise time of evoked α5IA-sensitive currents with that of their controls. We obtained α5IA-sensitive currents by subtracting evoked currents in the presence of the drug from their controls (Fig. 2*d*, inset). Both controls and α5IA-sensitive evoked currents had similar kinetics, indicating that α5-containing $GABA_A$Rs are most probably located at postsynaptic sites [Fig. 2*d*, inset; $t_{20–80\%} = 3.43 \pm 0.43$ and $t_{20–80\%} = 4.00 \pm 0.92$ ms, respectively ($n = 6$); $P > 0.05$]. In addition, no tonic current was revealed during the application of the drug in young and older mice (-0.21 ± 0.97 and 0.17 ± 0.56 pA at PN7–11 and PN21–29; $P > 0.05$). Overall, our pharmacological analyses confirmed the single-cell RT–PCR data, demonstrating the presence of functional α5-containing $GABA_A$Rs in a proportion of interneuron–NG2 cell synapses at early stages of postnatal development.

Postnatal Down-Regulation of the γ_2 Subunit in Cortical NG2 Cells

Most synaptic $GABA_A$Rs in neurons are assembled from α and β subunit variants in combination with the γ2 subunit, which is an important subunit for the clustering of receptors at the postsynaptic site (Essrich et al. 1998; Schweizer et al. 2003). If γ2 constitutes an important molecular component of synaptic receptors in neocortical NG2 cells, the loss of synaptic activity in these cells might be accompanied by its down-regulation. In line with this hypothesis, the γ2 subunit mRNAs were expressed in around 45% of the cells at PN7–11 and virtually disappeared at PN21–29 (Fig. 1*c*,*d*,*g*). To test whether the postnatal drop of γ2 expression occurs in functional $GABA_A$Rs of NG2 cells, we bath-applied diazepam, a benzodiazepine known to act preferentially at the interface between most α subunits and the γ2 subunit, during recordings of evoked currents (Pritchett et al. 1989; Puia et al. 1992; Luddens et al. 1994). As expected from the expression levels of the γ2 subunit mRNAs in NG2 cells during the second postnatal week, the effect of this compound was very variable from cell to cell, including cells that were insensitive (Fig. 3*a*,*c*). Nevertheless, diazepam significantly increased, in average, the amplitude of evoked GABAergic currents in the population of tested NG2 cells at PN7–11, when the frequency of spontaneous synaptic activity of these cells is high (Fig. 3*a*,*d*; see Vélez-Fort et al. 2010 for the frequency of synaptic events). On the contrary, diazepam failed to change significantly the evoked response amplitudes at PN21–29 when the frequency of spontaneous synaptic activity reaches minimal levels (Fig. 3*b*,*d*; see Vélez-Fort et al. 2010 for the frequency of synaptic events).

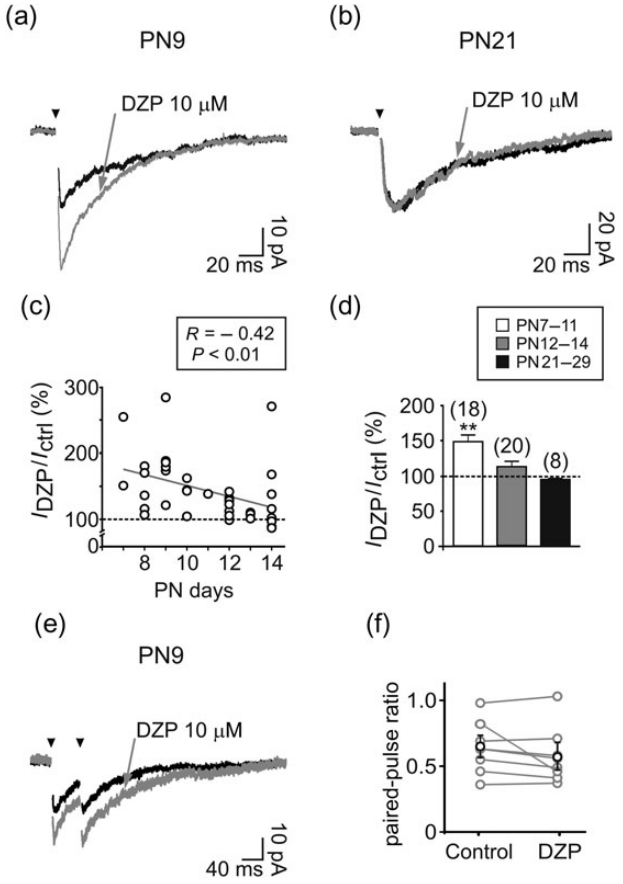

Figure 3. Effect of diazepam on evoked $GABA_A$R-mediated currents of cortical NG2 cells. (*a* and *b*) Effect of diazepam (DZP, gray traces) on evoked $GABA_A$R-mediated currents elicited in 2 NG2 cells held at −70 mV at PN9 (*a*) and PN21 (*b*). (*c*) Relationship between the percentage of amplitude increase induced by diazepam in evoked currents and postnatal days for individual cells. The gray solid line indicates a linear regression fit with a negative slope. (*d*) Histogram comparing the effect of 10 μM diazepam in NG2 cells recorded at PN7–11, PN12–14, and PN21–29. $^{**}P < 0.01$. (*e*) Paired-pulse stimulation of neuronal fibers elicited inward currents in a NG2 cell held at −70 mV at PN9. For all traces, stimulus artifacts were blanked for visibility and the time of stimulation is indicated with a black arrowhead. (*f*) Plot comparing the paired-pulse ratio obtained in control and in the presence of diazepam for 8 NG2 cells sensitive to this agent in young mice. The paired-pulse ratios of currents obtained from individual cells are in gray and of averaged ratios in black.

Although γ2 is probably not the only γ subunit at interneuron-NG2 synapse, our results strongly suggest that this subunit is an important component of synaptic $GABA_A$Rs of neocortical NG2 cells at PN7–11. Nevertheless, it was recently shown that $GABA_A$Rs in the developing brain could be located at presynaptic sites where they exert a depolarizing effect that increases the release probability of GABA (Trigo et al. 2007). The effect of diazepam in young mice could, thus, be due to the modulation of presynaptic $GABA_A$Rs enhancing GABA release from interneurons rather than to a direct effect on NG2 cell receptors. To examine potential effects of diazepam on the release probability, we performed paired-pulse stimulation of neuronal fibers. As expected for a direct postsynaptic effect of diazepam, the paired-pulse ratio of evoked $GABA_A$R currents remained unchanged in cells sensitive to diazepam (Fig. 3*e*,*f*).

These results indicate that diazepam directly affects postsynaptic receptors in NG2 cells, confirming that the functional expression of γ2 in these cells at PN7–11 is down-regulated during postnatal development.

Diazepam can also potentiate some $GABA_A$Rs containing γ1 and γ3 (Knoflach et al. 1991; Olsen and Sieghart 2009). We thus used zolpidem, a molecule that binds receptors specifically at the interface between α1 and γ2 at low concentrations (Pritchett and Seeburg 1990; Wafford et al. 1993; Buhr and Sigel 1997) to confirm the postnatal down-regulation of γ2 and to better define the subunit expression of $GABA_A$Rs in NG2 cells. As for diazepam, the effect of 1 μM zolpidem was variable from cell to cell with 2 of 8 cells being insensitive to this compound at PN7–11 (Fig. 4*e*). Nevertheless, on average, 1 μM zolpidem increased the amplitude of evoked GABAergic currents at PN7–11, corroborating the presence of $GABA_A$Rs containing the γ2 subunit at this age (Fig. 4*a*,*d*). The effect of zolpidem also identifies the α1 subunit as an important molecular component of these receptors in NG2 cells of the second postnatal week. As expected for older mice, whose responses are exclusively extrasynaptic, 1 μM zolpidem did not affect evoked GABAergic currents in NG2 cells (Fig. 4*b*,*d*). Since zolpidem insensitivity in older mice may be due to the lack of either α1 or γ2, we increased the concentration to 10 μM. Indeed, high concentrations of this molecule enhance receptor currents containing other α subunits together with γ2 (Pritchett and Seeburg 1990; McKernan et al. 1991). Zolpidem at 10 μM did not potentiate evoked $GABA_A$R currents, supporting the lack of γ2-containing $GABA_A$Rs in older animals (Fig. 4*c*,*d*). It is also noteworthy that no tonic current was revealed during the application of either diazepam or zolpidem throughout development (−1.28 ± 1.09 and −0.99 ± 0.69 pA at PN7–11 and PN21–29 for 10 μM diazepam; 0.87 ± 3.02 pA at PN7–11 for 1 μM zolpidem and 0.63 ± 5.23 pA at PN21–29 for 10 μM zolpidem; $P > 0.05$, in all cases). In conclusion, our molecular and pharmacological data show that, in addition to α5, both α1 and γ2 are important molecular components of functional synaptic $GABA_A$Rs of NG2 cells at PN7–11.

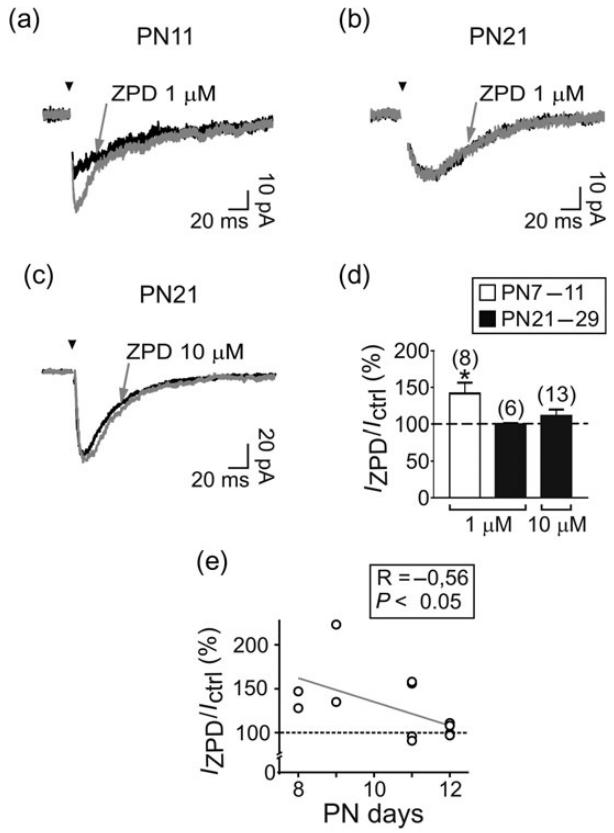

Figure 4. Effect of zolpidem on evoked $GABA_A$R-mediated currents of cortical NG2 cells. (*a*–*c*) Effect of zolpidem (ZPD; gray traces) on evoked $GABA_A$R-mediated currents elicited in 3 NG2 cells held at −70 mV at PN11 (*a*), PN21 (*b*), and PN21 (*c*). For all traces, stimulus artifacts were blanked for visibility and the time of stimulation is indicated with a black arrowhead. (*d*) Histogram comparing the effect of zolpidem at different concentrations in NG2 cells recorded at PN7–11 and PN21–29. $^*P < 0.05$. (*e*) Relationship between the percentage of amplitude increase induced by zolpidem in evoked currents and postnatal days for individual cells. The gray solid line indicates a linear regression fit with a negative slope.

Postnatal Down-Regulation of the γ_2 Subunit in NG2 Cells Accompanies the Switch From a Synaptic to an Extrasynaptic GABAergic Transmission Mode

Spontaneous synaptic activity of cortical NG2 cells, which is predominantly GABAergic, appears at around PN3, is maximal between PN7 and PN11, then dramatically decreases at the end of the second postnatal week to reach minimal levels at around PN18 (Vélez-Fort et al. 2010). This temporal course reflects the reduction of GABAergic synaptic connectivity of NG2 cells at the end of the second week and its complete loss later in development. However, GABAergic currents were easily evoked by neuronal fiber stimulation and had similar amplitudes at all postnatal days, including just after PN11 when synaptic activity already declines [amplitude of evoked currents: −44.7 ± 4.9 and −56.8 ± 6.5 pA at PN7–11 ($n = 40$) and PN12–14 ($n = 28$), respectively; $P > 0.05$]. These observations strongly suggest that, as early as PN12, the drop of synaptic connectivity in NG2 cells coincides with the switch of transmission modes from synaptic to extrasynaptic. This is supported by slower rise times of evoked currents at PN12–14 compared with PN7–11 [rise times: 2.42 ± 0.31 and 3.29 ± 0.30 ms at PN7–11 ($n = 18$) and PN12–14 ($n = 17$), respectively; $P < 0.05$]. In addition, despite the variability on the diazepam effect, a clear negative correlation was observed between the percentage of current amplitude increase induced by this molecule and postnatal days during the second postnatal week and no significant increase of the evoked current in average at PN12–14 ($R = -0.42$; $P < 0.01$; Fig. 3*c*,*d*). Similarly to diazepam, a negative correlation was observed between the percentage of current amplitude increase induced by zolpidem and postnatal days during the second postnatal week ($R = -0.56$; $P < 0.05$; Fig. 4*e*). These data support a down-regulation of γ2 in concomitance with the decline of synaptic innervation and switch of transmission. It is noteworthy that Na^+ current densities, reported to decrease when NG2 cells undergo cell differentiation (De Biase et al. 2010; Kukley et al. 2010), were not significantly different between PN7–11 and PN12–14, suggesting that the loss of synaptic transmission in NG2 cells at the end of the second postnatal week is not influenced by a different state of differentiation (Supplementary Fig. 2; see Materials and Methods).

To demonstrate that the switch of transmission mode coincided with the drop of γ2 expression after PN7–11, we examined if evoked currents lose their synaptic nature during the second postnatal week. For this, we evaluated whether or not evoked currents originated from a synaptic GABA release

at interneuron–NG2 cell synapses by replacing extracellular Ca^{2+} with 5 mM Sr^{2+}. Indeed, Sr^{2+} desynchronizes vesicular release in presynaptic terminals allowing for the isolation of evoked GABAergic asynchronous miniature currents exclusively at synaptically connected cells (Goda and Stevens 1994; Vélez-Fort et al. 2010). As expected for evoked currents resulting from an exocytotic GABA release, a large decrease in the amplitude occurred in the presence of Sr^{2+} in all tested cells (Fig. 5*a*,*b*). Asynchronous miniature events were observed in all recorded NG2 cells of the second postnatal week at variable frequencies, ranging from 0.08 to 1.5 Hz (mean frequency: 0.55 ± 0.12; $n = 16$; Fig. 5*c*,*d*). Nevertheless, a linear correlation was observed between the amplitude of evoked miniature currents and postnatal days ($R = 0.69$; $P < 0.025$; Fig. 5*c*–*e*),

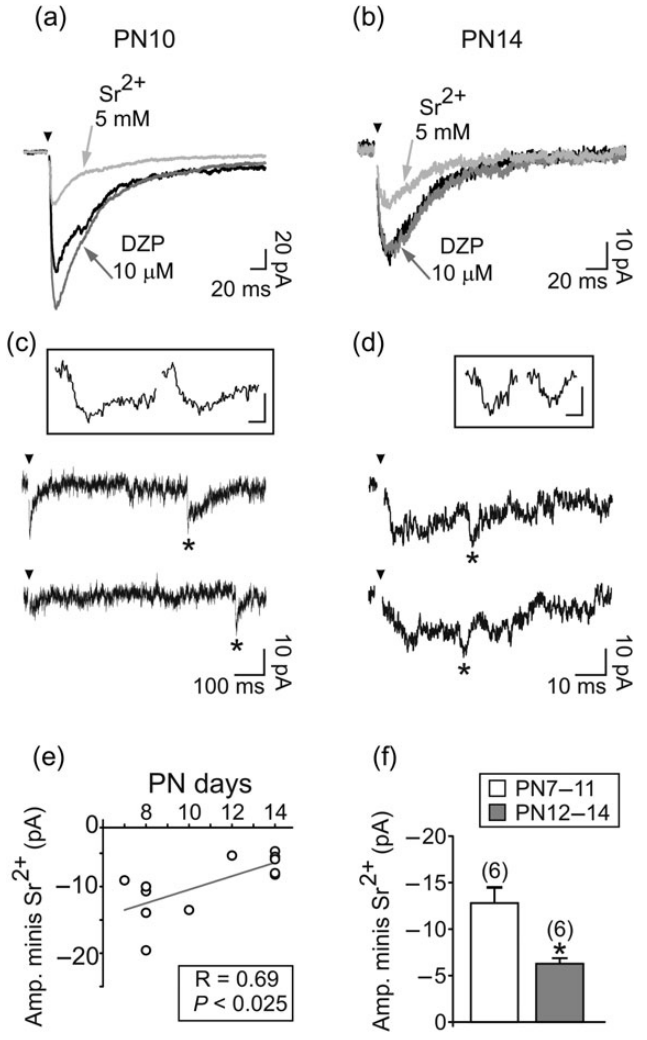

Figure 5. Postnatal down-regulation of γ_2 subunit expression in NG2 cells correlates with the loss of synaptic activity and the switch of transmission mode. (*a* and *b*) Averaged traces of evoked currents of 2 NG2 cells held at −70 mV at PN10 (*a*) and PN14 (*b*) in control (black), in the presence of diazepam (dark gray) and when 2 mM Ca^{2+} was replaced by 5 mM Sr^{2+} (light gray). Note that currents were strongly decreased in the presence of Sr^{2+} in both cells. (*c* and *d*) Single traces of evoked currents in 5 mM Sr^{2+} of the NG2 cells shown in *a* and *b*. The insets illustrate individual currents at higher resolution (asterisk). Note the amplitude reduction of evoked miniature events in (*d*, inset). Inset scale bars: 10 pA, 2 ms. (*e*) Relationship between the amplitude of evoked asynchronous miniature currents and postnatal days for individual cells. The solid line indicates a linear regression fit with a positive slope. (*f*) Histogram comparing the amplitude of evoked miniature currents of NG2 cells recorded in Sr^{2+} at PN7–11 and PN12–14. *$P < 0.05$.

showing a strong reduction in the expression of functional $GABA_A$Rs at postsynaptic sites at the end of this period. Indeed, evoked miniature currents had significantly different peak amplitudes between PN7–11 and PN12–14 (Fig. 5*f*). Since, in control conditions, the amplitude of evoked currents remained unchanged between these 2 early developmental stages, the amplitude decrease of miniature currents confirms that, at PN12–14, evoked responses are not only synaptic, but have already an extrasynaptic component. Moreover, this transition from synaptic to extrasynaptic is complete at PN21–29, since evoked miniatures events in Sr^{2+} recorded in the presence of diazepam were infrequent at this stage (mean frequency: 0.09 ± 0.03 Hz; $n = 8$).

We demonstrate that the down-regulation of the γ2 subunit in NG2 cells begins at the end of the second postnatal week in concomitance with the decline of synaptic innervation and the change of transmission mode from synaptic to extrasynaptic. These results set γ2 as an important molecular determinant in the change of transmission modes between interneurons and NG2 cells in the somatosensory cortex during development.

Discussion

Significant developmental changes of $GABA_A$R subunit expression have been described in the brain for more than 2 decades. Age-dependent modifications in subunit expression have mainly been associated with maturation of synaptic function in neurons, disregarding possible effects on glial cells and their progenitors. The expression of these receptors by NG2 cells has also been recognized already 2 decades ago (Bergles et al. 2010; Velez-Fort et al. 2012), but postnatal regulation of $GABA_A$R subunits in these cells had not been yet evaluated. Single-cell RT–PCR, a powerful and sensitive technique that allows for the analysis of transcript expression in patched cells, was used in this report to provide, specifically in NG2 cells, a comprehensive picture of the developmental expression pattern of main cortical $GABA_A$R subunits. Interestingly, the temporal expression profile of several subunits in these cells exhibits a similar developmental regulation as observed in the neocortex (Laurie et al. 1992; Golshani et al. 1997). Similar to subunit expression in NG2 cells, α1, α2, α5, β1, β2, β3, and γ1 mRNAs were more abundant in cortical regions during the first 2 postnatal weeks (Laurie et al. 1992). Then, α2, α5, and β1 transcripts decrease, while those of α1, β2, β3, and γ1 remain high in the adulthood (Laurie et al. 1992). In addition, δ mRNA appears weakly express in NG2 cells at PN21–29 as well as in layer V of the adult neocortex (Wisden et al. 1992). Despite these similarities on expression patterns, α3, α4, γ2, and γ3 have cell type-specific temporal expression profiles in NG2 cells that differ from those in the total neocortex. Transcripts for α3 and α4 are low in NG2 cells at PN7–11 and increase with age, whereas in the young total neocortex α3 peaks and α4 already reaches adult levels at this early developmental stage (Laurie et al. 1992). Other notable differences concern γ2 and γ3 subunits. While γ3 mRNA is frequently detected in NG2 cells between the second and fourth postnatal weeks, in the total neocortex it reaches a peak of expression at P12 and declines thereafter. Finally, γ2, which is invariably present in the neocortex from the second postnatal week (Laurie et al. 1992), undergoes a drastic drop of expression in NG2 cells at the end of this week. This cell type-specific postnatal regulation of $GABA_A$R subunits indicates that NG2 cells have their own

expression program, conferring specific pharmacological and physiological profiles to $GABA_AR$-mediated evoked responses.

Pharmacological analyses confirmed our single-cell RT–PCR data and allowed us to conclude that at least functional α1- and α5-containing $GABA_ARs$ in the barrel cortex of young mice are present at most interneuron–NG2 cell synapses. This is different to hippocampal NG2 cells, where α5 is less prevalent (Passlick et al. 2013). Considering that α1 and α5 do not seem to be associated in single native receptors (McKernan et al. 1991), at least 2 different receptor pools probably coexist in NG2 cells during early cortical development. This idea is strengthened by single-cell RT–PCR analyses, showing that 6 of 10 cells expressing the α1 subunit also express α5. Although α5 is better known as an extrasynaptic subunit (Farrant and Nusser 2005), α5-containing $GABA_ARs$ in NG2 cells partly mediate evoked responses in NG2 cells of young mice when synaptic transmission is predominant and disappear almost completely in the fourth postnatal week when transmission does not rely on functional synapses, but involves solely GABA spillover (Fig. 2*d–f*; Vélez-Fort et al. 2010). The presence of α5-containing receptors at hippocampal interneuron–NG2 cell synapses has been previously suggested by the sensitivity for diazepam and the lack of effect of zolpidem on GABAergic miniature synaptic currents (Lin and Bergles 2004), although transcript analysis rarely detected the subunit in these NG2 cells (Passlick et al. 2013). In addition, a pool of synaptic α5-containing receptors has been previously described in dendrites of cortical and hippocampal pyramidal neurons (Serwanski et al. 2006; Ali and Thomson 2008). Therefore, despite the widely accepted role of α5 in neuronal tonic inhibition (Caraiscos et al. 2004), the localization of this subunit is not exclusively extrasynaptic and can be found at postsynaptic sites in neurons as well as in neocortical NG2 cells. Interestingly, Ali and Thomson (2008) demonstrated that α1- and α5-containing $GABA_ARs$ are both expressed by neocortical pyramidal cells, but located at different neuronal postsynaptic sites. Moreover, the specific localization of these receptors in neurons is input-dependent, since synaptic responses to presynaptic bitufted interneurons are mediated by α5-containing receptors, whereas those to presynaptic parvalbumin-positive, fast-spiking cells are mediated by α1-containing receptors. By analogy, the presence of distinct postsynaptic $GABA_ARs$ on NG2 cell membranes of young mice raises the possibility that distinct classes of presynaptic interneurons are synaptically connected to neocortical NG2 cells, the identity of which are still elusive.

Concerning α4-containing receptors, the lack of the effect of furosemide on evoked GABAergic responses of NG2 cells in the fourth postnatal week did not completely rule out whether this subunit is present or not in extrasynaptic $GABA_ARs$. According to pharmacological and molecular analyses, the most likely γ subunits expressed in neocortical NG2 cells of older mice are γ1 and/or γ3. Indeed, the insensitivity to diazepam and zolpidem at later stages, even at high concentrations, and the very infrequent expression of γ2 and δ mRNAs are in line with the idea that γ1 and/or γ3 are very abundant and potential partners of the α4 subunit. Since the pharmacology of α4 has been defined by investigating α4γ2 and α4δ-containing receptors, it may not be valid for receptor combinations containing other γ subunits. In addition, the α4 subunit is activated by ambient GABA, mediating tonic inhibition (Chandra et al. 2006) rather than by local GABA spillover from nearby synapses. Here, we showed that tonic currents in neocortical NG2 cells are very small in young animals and virtually absent in the fourth postnatal week, suggesting that most $GABA_ARs$ of NG2 cells are synaptic or very close to synapses. However, we cannot exclude that ambient GABA induces a α4-mediated tonic response under conditions in which GABA increases at pathological levels (Cope et al. 2009). Interestingly, hippocampal NG2 cells display a more prominent tonic GABA responses (Passlick et al. 2013), suggesting that the subunit composition and/or subcellular distribution of $GABA_ARs$ in NG2 cells varies across brain regions.

A major finding of this report is the down-regulation of the γ2 subunit of postsynaptic $GABA_ARs$ in NG2 cells that accompanies the postnatal switch from synaptic to extrasynaptic GABAergic transmission between interneurons and these cells in the barrel cortex. This is consistent with the role of this subunit described in neurons in receptor clustering and recruitment of the submembrane scaffold protein gephyrin to postsynaptic sites (Essrich et al. 1998; Schweizer et al. 2003). Specifically, our pharmacological analyses point toward a decline in γ2 expression from P12, as indicated by the strong reduction in the sensitivity to the benzodiazepine receptor agonist, diazepam, at a time point when spontaneous synaptic activity is largely decreased, but can still be detected in NG2 cells (Fig. 3; Vélez-Fort et al. 2010). Changes in subunit assembly are thus triggered in NG2 cells in association with that of transmission mode. Our data also reconcile controversial results on the molecular composition of $GABA_ARs$ of cultured oligodendrocyte precursor cells. Indeed, whereas Von Blankenfeld et al. (1991) suggested the presence of functional γ2-containing receptors, Bronstein et al. (1998) and Williamson et al. (1998) claimed that this subunit is not expressed in these cells. In the present report, we demonstrated that native receptors of NG2 cells are heterogeneous with regard to the expression of this subunit.

Recently, it was proposed that the transition of NG2 cells to oligodendrocytes is concomitant with the removal of synaptic input and the down-regulation of Na^+ channel expression (De Biase et al. 2010; Kukley et al. 2010). It can be hypothesized that NG2 cells with $GABA_ARs$ lacking the γ2 subunit at the end of the second postnatal week correspond to cells in a more differentiated state. However, the presence of synaptic activity and large Na^+ currents in NG2 cells less sensitive to diazepam suggests that the switch of γ2 expression is not influenced by the state of cell differentiation, but rather depends on a postnatal developmental program. In addition, we recently demonstrated a divergence between physiological properties of NG2 cells at the second and the fourth postnatal weeks that are determined by the postnatal upregulation of Kir4.1 potassium channels (Maldonado et al. 2013). Developmental changes of membrane properties, molecular composition of $GABA_ARs$, and GABAergic transmission modes in NG2 cells reinforce the concept that these cells play distinct roles in neuronal networks in young versus older mice.

Compared with the glutamatergic system, only a limited description of GABAergic synaptic properties of NG2 cells exists, and thus, this field of research remains largely unexplored. The present report provides a framework for further research aiming at deciphering the role of GABAergic synapses in NG2 cells. In particular, α1, α5, and γ2 are interesting targets for specific deletion or manipulation in NG2 cells early in

postnatal development. Interestingly, GABA promotes the differentiation of neuronal progenitors, presumably through the action of direct synaptic inputs from interneurons in the hippocampus (Tozuka et al. 2005). A similar mechanism is conceivable for NG2 cells.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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