Expression of Distinct \( \alpha \) Subunits of GABA\(_A\) Receptor Regulates Inhibitory Synaptic Strength

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Distinct \( \alpha \) subunit subtypes in the molecular assembly of GABA\(_A\) receptors are a critical determinant of the functional properties of inhibitory synapses and their modulation by a range of pharmacological agents. We investigated the contribution of these subunits to the developmental changes of inhibitory synapses in cerebellar granule neurons in primary cultures from wild-type and \( \alpha 1 \) subunit \(-/-\) mice. The decay time of miniature inhibitory postsynaptic currents (mIPSCs) halved between 6 days in vitro ( DIV6) and DIV12. This was paralleled by the decrease of \( \alpha 2 \) and \( \alpha 3 \) subunits, the increase of \( \alpha 1 \) and \( \alpha 6 \) subunits expression at synapses, and changes in the action of selective \( \alpha \) subunit modulators. A small but significant shortening of mIPSCs was observed with development in cells from \(-/-\) mice together with a decrease in the expression of \( \alpha 3 \) subunit. In contrast, the expression of \( \alpha 2 \) subunit at inhibitory synapses in \(-/-\) cells was significantly higher than in \(+/+\) cells at DIV11-12. \( \alpha 5 \) subunit was not detected, and increased sensitivity to a selective \( \alpha 4/\alpha 6 \) subunit agonist suggests increased expression of extrasynaptic receptors in \(-/-\) mice. \( \beta 2/\beta 3 \) subunit expression and loreclezole sensitivity increased with development in \(+/+\) but not in \(-/-\) cells, supporting the preferential association of the \( \alpha 1 \) with the \( \beta 2 \) subunit. Synaptic charge transfer strongly decreased with development but was not different between cells in the \(+/+\) and \(-/-\) groups until DIV11-12. Our results uncover a pattern of sequential expression of \( \alpha \) subunits underlying the changes in functional efficacy of GABAergic networks with development.

INTRODUCTION

The efficacy of inhibitory neurotransmission in the brain is strongly dependent on kinetics of the postsynaptic receptor-channels. GABA\(_A\) receptor, one of the main inhibitory receptor-channels in the brain, exhibits a variety of distinct kinetic behaviors. Subunit composition of GABA\(_A\) receptors determines channel kinetics and pharmacological sensitivity. \( \alpha 1 \) subunit, for example, produces fast current decay and confers sensitivity to the imidazopyridine, zolpidem (Vicini et al. 2001), \( \alpha 2 \) and \( \alpha 3 \) prolong deactivation (Gingrich et al. 1995; Lavoie et al. 1997; McClellan and Twyman 1999; Verdoorn 1994), and \( \alpha 6 \) confers sensitivity to furosemide (Korpi et al. 1995) and is responsible for tonic inhibition (Hamann et al. 2002; Kaneda et al. 1995; Wall and Usowicz 1997). In addition, a GABA agonist 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridin-3-ol (THIP), has been shown to act as a superagonist at extrasynaptic \( \alpha 4 \) and \( \delta \) subunit-containing receptors (Brown et al. 2002) that are preferentially enhanced by ethanol (Wallner et al. 2003).

In cerebellar neurons of mice and rats, expression of \( \alpha 1 \) and \( \alpha 6 \) subunit mRNA increases with age, whereas \( \alpha 2 \) and \( \alpha 3 \) subunit mRNA is downregulated (Laurie et al. 1992a,b; Poulter et al. 1992). Consequently, the kinetics of spontaneous and miniature IPSCs (sIPSCs and mIPSCs) in cerebellar granule cells (CGCs) become faster with development as demonstrated by electrophysiological recordings in slices (Brickley et al. 1996; Tia et al. 1996). This is due to increased expression of \( \alpha 1 \) but not \( \alpha 6 \) subunits as shown in mutant mice lacking these subunits (Farrant et al. 1999; Vicini et al. 2001). Development of an in vitro system allows direct correlation between anatomical (Gao and Fritschy 1995) and functional approaches to study inhibitory synapses, but it has not yet been established whether developmental changes in inhibitory synaptic function observed in vivo can be reliably replicated in culture conditions. Primary cultures of CGCs are best maintained in a depolarizing medium containing 25 mM KCl (Gallo et al. 1987), which, however, prevents detection of synaptic activity with electrophysiological methods (Mellor et al. 1998). In this culture condition, immunocytochemical studies have shown some clustering of \( \alpha 1 \) but not \( \alpha 3 \) or \( \alpha 6 \) subunits (Gao and Fritschy 1995; Studler et al. 2002). Low (5 mM) concentration of K\(^+\) in a medium supplemented with specific additives favors formation of both inhibitory (Mellor et al. 1998; Ueno et al. 1996; Virginio et al. 1995) and excitatory (Chen et al. 2000; Losi et al. 2002) networks between CGCs and GABAergic interneurons in culture. This allows electrophysiological recordings from such cultures. sIPSCs and mIPSCs under these conditions can be recorded several days after plating, but have only been extensively characterized late in development (≥12 days in vitro (DIV12)) (Mellor et al. 2000). In these cultures, whole cell recordings revealed tonic GABA-mediated current that is TTX sensitive and therefore mostly mediated by spill-over of GABA from high-density active inhibitory synaptic terminals (Leao et al. 2000). This tonic current is absent in CGCs in slices from mice with deletion of the \( \alpha 6 \) or \( \delta \) subunits (Brickley et al. 2001; Stell et al. 2003).

We investigated age-dependent changes of GABA\(_A\)-mediated IPSCs and tonic current in CGC cultures. The data gathered with electrophysiological, immunocytochemical, and pharmacological techniques applied to CGCs cultured from wild-type (+/+ ) and \( \alpha 1 \) knockout (−/−) mice demonstrate...
specific contributions of distinct α subunits underlying developmental changes of iPSC kinetics in vitro.

METHODS

Mutant mice and genotyping

α-subunit-deficient mice were produced at the University of Pittsburgh as described (Vicini et al. 2001) and shipped to Georgetown University for breeding and all experimental procedures. Heterozygous mice on a mixed genetic background (C57BL/6J, strain 129/Sv, FVB/N) were interbred to produce wild-type (+/+), heterozygous (−/+), and homozygous (−/−) knockout mice. Polymerase chain reaction (PCR) was used to genotype mutant mice. Total genomic DNA was isolated at the third postnatal day from tail snips with DNeasy Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. DNA was amplified with PCR using the primers GAB1 (tcgtcagttgacaatagc), GAB2 (acgcat-acctctcttgg), and GAB3 (ttgattcttgcatagatg). GAB1 and GAB2 amplify across the deleted region of α1 DNA sequence and allow identification of the wild-type allele; GAB1 and GAB3 amplify outside the deleted sequence and allow identification of the knock-out allele. Primers (2 μl each) and DNA (5 μl) were mixed with Ready-to-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ) and diluted to 25 μl with distilled H2O. Twenty-seven PCR cycles were run after initial denaturation at 95°C for 5 min. Each cycle consisted of: denaturation at 95°C for 1 min, primer annealing at 57°C for 1.5 min, and primer elongation at 72°C for 2 min. A final elongation step was performed at 72°C for 3 min. PCR amplification products were loaded at 10 μl/lane into a 2% agarose gel, separated by electrophoresis, and visualized with ethidium bromide staining.

CGC cell culture and immunocytochemistry

Primary cultures of mouse CGCs were prepared from postnatal day 5–7 mice. Mouse pups were killed by decapitation in agreement with National Institutes of Health guidelines for the treatment of laboratory animals (Public Health Service) and were perfused with PBS. Primary cultures of mouse CGCs were prepared from postnatal day 5–7 mice. Mouse pups were killed by decapitation in agreement with National Institutes of Health guidelines for the treatment of laboratory animals (Public Health Service) and were perfused with PBS. All immunostaining experiments were carried out at room temperature. Fixed neurons with Alexa488-conjugated secondary antibodies (Molecular Probe; 1:1,000).

Neurons were imaged on a Nikon E600 microscope (Nikon) equipped with ×60, 1.0 N.A. objective. Nikon band-pass filter cubes were used for Cy3 or Alexa488 fluorescence. Digital images were acquired with a CFW-1310 (Scion, Frederick, MD), 10-bit (1,024 gray scale intensity level) CCD digital camera, 1,360 × 1,024 pixel array. Images were analyzed with MetaMorph (Universal Imaging, Downingtown, PA), and pseudocolored for presentation with Adobe Photoshop 7.0 (Adobe, San Jose, CA). Antibody staining was considered positive when staining intensity was at least twice the fluorescence intensity of the background. For quantification of developmental changes of synaptic density, the number of GAD-positive puncta was counted along 100-μm segments of randomly selected dendrites. At least 12 segments were analyzed at each age. For the analysis of synaptic localization of α subunits, the percent of GAD-positive puncta containing α subunit-specific staining along 100-μm segments of randomly selected dendrites was counted (>10 segments for each subunit). Synaptic localization was assumed when the center pixels of subunit clusters and GAD puncta were <1 μm apart. Statistical comparisons were done using a two-tailed Student’s t-test assuming homogeneity of variances of the samples with Bonferroni corrections. Data values are expressed as means ± SE.

Electrophysiology

Coverslips with CGCs were placed on the stage of an inverted microscope (TM2000, Nikon) equipped with fluorescent and phase contrast optics. All recordings were performed at room temperature (24–26°C) from neurons maintained for 6–14 DIV. Continuously perfused extracellular solution contained (in mM) 145 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 5 HEPES, 5 glucose, and 15 sucrose and 0.25 mg/l phenol red and 10 μM α-serine (all from Sigma) adjusted to pH 7.4 with NaOH. Electrodes were pulled in two stages on a vertical pipette puller from borosilicate glass capillaries (Wiretrol II, Drummond, Broomall, PA) and filled with recording solution containing (in mM) 145 KCl, 10 HEPES, 5 ATP Mg, 0.2 GTP Na, and 10 BA, adjusted to pH 7.2 with KOH. Pipette resistance was 5–8 MΩ. Whole cell voltage-clamp recordings were made at −60 mV with an Axopatch-1D amplifier (Axon Instruments, Union City, CA), and access resistance was monitored throughout the recordings. Capacitance was calculated from a transient current response to a hyperpolarizing 10-mV pulse. Currents were filtered at 1 kHz with an 8-pole low-pass filter (Frequency Devices, Haverhill, MA), digitized at 5–10 kHz using an IBM-compatible microcomputer equipped with Digidata 1322A data-acquisition board and pCLAMP9 software (both from Axon Instruments). Off-line data analysis, curve fitting, and figure preparation were performed with Clampfit 9 software. GABA, THIP, flunitrazepam, furosemide, zolpidem, brentazolin, bicuculline metabotroide (BMR, Sigma), and lorecetol (a gift from Janssen Research Foundation, Beerse, Belgium) were dissolved either in water or in dimethylsulfoxide (DMSO, <0.001% final concentration, Sigma) and diluted in the extracellular medium. Drugs were locally applied means of a Y tube (Murase et al. 1989). mIPSCs were isolated by application of 0.5 μM tetrodotoxin (TTX, Alamone Labs, Israel). Infraguent AMPA-mediated miniature excitatory postsynaptic currents (mEPSCs) were easily identified by the very rapid decay (<2 ms) and excluded from the analysis. mIPSC and mEPSC averages were based on ≥50 events in each cell studied. The decay phase of currents was fitted using a simplex algorithm for least-squares exponential fitting routines with triple exponential equation of the form I(t) = I1 * exp(−t/τ1) + I2 * exp(−t/τ2) + I3 + exp(−t/τ3), where I1 is a peak amplitude of a decay component and τ1 is the corresponding decay time constant. To allow for easier comparison of decay times between experimental conditions, the three decay time components were combined into a weighted time constant rw = [I1/(I1 + I2 + I3)] * τ1 + [I2/(I1 + I2 + I3)] * τ2 + [I3/(I1 + I2 + I3)] * τ3. Mean current changes
with BMR and TTX were performed by computing the differences between 2 averages of baseline current that were devoid of sIPSCs before and during applications of each drug. All data are expressed as mean ± SE, unless otherwise indicated; P values represent the results of 2-tailed Student’s t-test with Bonferroni corrections.

RESULTS

Spontaneous and miniature IPSCs in developing CGCs cultures

sIPSCs were studied in mouse CGCs in primary cultures with whole cell voltage-clamp recordings. Granule neurons were identified as neurons with a cell body axis of ≤7 μm that had relatively simple processes and lacked sustained high-frequency action potential firing, a hallmark of GABAergic interneurons. Cells were voltage-clamped at −60 mV using an intracellular pipette solution containing symmetrical chloride to the bath solution and were observed as inward currents (Fig. 1). As reported previously, culture medium containing 5 mM KCl allows the formation of functional inhibitory and excitatory synapses (Chen et al. 2000; Mellor et al. 1998, 2000; Virginio et al. 1995). We began our studies in cells cultured for 6 days in vitro (DIV6), 24 h after lowering K⁺ concentration in the medium. sIPSCs occurred in the majority of cells investigated at this age with highly variable frequency of occurrence and amplitude. Examples of sIPSCs recorded from control cells at DIV6 and DIV9 are reported in Fig. 1A. The decay of sIPSCs in these cells was best described by three exponential curves (Fig. 1A), similar to sIPSCs recorded from CGCs in cerebellar slices (Farrant and Brickley 2003). The three-component decay was maintained when current flow was reverted by clamping the cell at +60 mV (not shown).

sIPSCs of CGCs became steadily faster with development in culture (Fig. 1C). The weighted time constant (τw) based on a triple-exponential fit of decay (see METHODS) was 35.5 ± 1.7 ms (n = 33) at DIV6-7 and decreased to 18.2 ± 1 ms (49%) by DIV11-12 (n = 34, P < 0.01). To investigate the role of the α1 subunit in developmental changes of sIPSCs, we cultured CGCs from α1 −/− mice. Developmental speeding of sIPSCs in these cells could be observed but was less pronounced (Fig. 1B). sIPSCs averaged 36.7 ± 1.7 ms (n = 36) at DIV6-7 and decreased to 30.6 ± 1.9 ms (17%) by DIV11-12 (n = 24, P < 0.01). The τw of sIPSC averages in the +/+ group were significantly faster than those of sIPSCs in the −/− group for all ages after DIV7 (P < 0.01). These data support the critical role of α1 subunit in mediating the developmental decrease of sIPSCs in CGC cultures first reported in cerebellar slices (Vicini et al. 2001).

sIPSCs in CGCs are generated by presynaptic action potentials in GABAergic interneurons and can be due to simultaneous activation of multiple synaptic sites. Analysis of mIPSCs, which are generated at single synaptic sites, allows for more robust conclusions about changes at individual synapses. mIPSCs were isolated by application of TTX (0.5 μM) and mIPSC averages fitted with three exponential curves. Data from consecutive days were grouped to increase statistical power (Fig. 2). Individual time constant values and their relative contributions to peak mIPSCs are reported in Table 1. The τw of mIPSC averages in both the +/+ and the −/− groups showed progressive developmental decrease (Fig. 2A). mIPSCs from the +/+ group were faster than those from the −/− group at all ages after DIV6-7 (P < 0.01) similar to what was observed for sIPSCs. The decrease in τw was accompanied...
by a decrease in peak amplitude in both genotypes (Fig. 2B, Table 1). τw changes were significant for the wild-type group at DIV11-12 and only reached significance between the two groups at DIV8-9. Charge transfer was significantly reduced in both the +/+ and the −/− groups as early as DIV8-9 (Fig. 2C), indicating that the decreased mIPSCs amplitude compensates for the slower decay in −/− CGCs. However, by DIV11-12, charge transfer in +/+ CGCs was smaller than in −/− CGCs (P < 0.05). The frequency of mIPSCs increased in both the −/− and the +/+ groups at DIV11-12 (P < 0.01 for −/−; P < 0.05 for +/+) and as early as DIV8-9 for the +/+ group (Fig. 2D). The differences were only significant between groups at DIV8-9.

These results clearly define a role for α1 subunit in developmental changes of IPSCs. However, changes in IPSCs in cells from α1 −/− mice suggest additional and/or compensatory mechanisms underlying GABA channel changes in developing CGCs.

**Pharmacology of mIPSCs in developing CGS**

We further assessed the involvement of other GABAA receptor subunit combinations in developmental changes of mIPSCs in CGCs from +/+ and −/− mice using pharmacological tools. We first studied prolongation of current decay by the imidazopyridine, zolpidem (200 nM), shown to preferentially enhance Cl− current to a similar extent in the +/+ and the −/− groups (P > 0.39, Fig. 3, A and B), confirming the developmental increase of α1 expression in wild-type CGCs (Fig. 3B).

α6 subunit has been previously shown to be developmentally regulated in cerebellar cultures grown in 5 mM KCl (Mellor et al. 1998). Participation of the α6 subunit in inhibitory synaptic transmission has likewise been demonstrated (Mellor et al. 2000; Tia et al. 1996). To assess if α1 subunit deletion affects the expression of α6 subunit at inhibitory synapses, we studied inhibition of sIPSCs in the +/+ and the −/− groups by furosemide (100 μM), a diuretic selective antagonist of GABAA receptors containing α6 subunits (Korpi et al. 1995). Application of furosemide at DIV11 minimally affected decay kinetics in both groups and reduced peak current to a similar extent in the +/+ and the −/− groups (P > 0.39, Fig. 3, C and D). Compared with DIV8, the percent reduction of the peak sIPSC amplitude was significantly greater at DIV11 in both genotypes (Fig. 3D). These findings agree with previous reports of developmental increase of α6 subunit in inhibitory synapses to CGCs in vivo (Tia et al. 1996) and show, in addition, that such increase is not markedly affected by α1 subunit deletion.

We also investigated tonic current recorded in CGCs by measuring mean baseline current in the presence and absence of BMR, TTX, and BMR + TTX (Leao et al. 2000). The average BMR-sensitive current was very small (<7 pA) among all ages and genotypes tested and was not statistically different from the TTX-sensitive current (P > 0.29). Thus in primary CGC cultures, tonic GABAA receptor activation is minimal in contrast to observations in slices (Kaneda et al. 1995; Rossi and Hamann 1998; Wall and Usowicz 1997), likely due to effective local perfusion that facilitates GABA removal. We, therefore, directly activated GABAA receptor with THIP (Fig. 4A), a superagonist at α4β3δ receptors but with considerable potency and efficacy also for α6 subunit-containing receptors.

**TABLE 1. Values of individual time constants from triple exponential fitting of mIPSC decay in CGCs from +/+ and α1 −/− mice**

<table>
<thead>
<tr>
<th>Days In Vitro</th>
<th>τ1 (ms)</th>
<th>τ2 (ms)</th>
<th>τ3 (ms)</th>
<th>τw (ms)</th>
<th>% τ2</th>
<th>% τ3</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+ (6–7)</td>
<td>6 ± 1.5</td>
<td>28 ± 4</td>
<td>104 ± 14</td>
<td>40 ± 3</td>
<td>52 ± 4</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>−/− (6–7)</td>
<td>3.1 ± 1.1</td>
<td>25 ± 3</td>
<td>92 ± 13</td>
<td>39 ± 2</td>
<td>53 ± 5</td>
<td>37 ± 6</td>
</tr>
<tr>
<td>+/+ (8–9)</td>
<td>3.6 ± 5</td>
<td>14 ± 2†</td>
<td>69 ± 7</td>
<td>21 ± 1†</td>
<td>50 ± 3</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>−/− (8–9)</td>
<td>2 ± 0.2*</td>
<td>19 ± 1</td>
<td>70 ± 6</td>
<td>31 ± 2†</td>
<td>50 ± 4</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>+/+ (11–12)</td>
<td>1.4 ± 0.2†</td>
<td>10 ± 1‡</td>
<td>40 ± 4‡</td>
<td>16 ± 1‡</td>
<td>60 ± 6</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>−/− (11–12)</td>
<td>2.8 ± 0.9</td>
<td>24 ± 5*</td>
<td>67 ± 25</td>
<td>31 ± 3*</td>
<td>49 ± 6</td>
<td>33 ± 7</td>
</tr>
</tbody>
</table>

Values are means ± SE, n > 22 in each group. r1–r3 are the decay time constants from decay fitting. %τ2 and %τ3 are the relative contribution of the respective components to the peak current amplitude. *P < 0.01 vs. wild type at the same days in vitro (DIV); †P < 0.01 vs. DIV6–7 within genotype. mIPSC, miniature inhibitory postsynaptic current; CGC, cerebellar granule cells.
minimal at inhibitory synapses in CGCs, it does not increase with development, and it does not change between genotypes. We also compared the prolongation of mIPSCs decay by the β2/β3-selective drug, loreclezole (Fisher et al. 2000; Wafford et al. 1994). Bath perfusion with 10 μM loreclezole similarly prolonged the τm in neurons of both genotypes at DIV7 (Fig. 4D). However, by DIV13 mIPSC prolongation of τm by loreclezole was significantly greater in +/- than in –/- CGCs. This result indicates that with development in culture, the expression of the β2/β3 subunit increases or the expression of β1 subunit decreases in CGCs from wild-type but not α1 knock out mice.

Immunohistochemistry

Immunofluorescence staining was used to correlate the electrophysiological results with directly visualized pattern of expression of α subunits at synaptic sites during development. Cultures of CGCs were immunostained using subunit specific antibodies and antibodies to glutamic acid decarboxylase (GAD)—a marker of GABAergic synapses. As illustrated in Fig. 5, CGCs, often clustered in small groups, form extensive neuritic networks. α1 subunit staining of CGCs at DIV6-7 and -11-12 from +/- mice revealed a strong increase of α1 immunoreactivity. Such staining was absent in CGCs from α1 –/- mice. In +/- cultures, a small population of larger neurons, possibly a subtype of GABAergic interneurons, displayed intense staining for the α1 subunits at all ages (not shown). Quantification of α1 subunit immunoreactivity at GABAergic synapses is described in the following text.

One striking feature of immunostaining was the formation of bright and well defined α subunit clusters. Smaller clusters and more diffused immunofluorescence similar to that previously reported in CGC cultures grown in high potassium were also present (Gao and Fritschy 1995; Studler et al. 2002). As can be observed in the high-magnification micrographs in Fig. 6, α1 and α2 subunit clusters were most dense along the dendrites but were observed also on cell bodies. We speculated that the punctate expression of α1 and α2 subunit clusters along the dendrites and cell bodies might correspond to areas of synaptic contact between the cells. Indeed, double staining of CGCs with antibodies against α1 subunit and GAD confirmed that a number of GAD-positive puncta were opposed to a postsynaptic α1 cluster at DIV8 in +/- CGCs (Fig. 6). Quantitative assessment of GAD/α subunit co-localization data supports

![Image](https://example.com/image.png)
these observations (see following text). Double staining with antibodies against α2 subunit and GAD showed that GAD-positive puncta were opposed to postsynaptic α2 subunit clusters in both +/+ and −/− CGCs (Fig. 6).

To further investigate the role of α subunits in developmental changes of inhibitory synapses in CGCs, we extended our study to α3, α5, and α6 subunits in developing +/+ and −/− CGCs. Immunolabeling with the α5 subunit at all ages was very faint as compared with all other subunits tested and without well-defined clustering, and it was not investigated further. As seen in Fig. 7, double staining with antibodies against distinct α subunits and GAD showed remarkable changes in punctate α1, α2, α3, and α6 subunit immunoreactivity with development. The density of GAD puncta per 16-μm dendritic length did not change with development, being on average 3.9 ± 0.4. The punctate expression of α3 and α2 subunits at GAD-positive sites was pronounced early in development. By DIV12, α2 subunit immunofluorescence became more diffuse, and well-defined clusters were preserved only at select few synapses (Fig. 7) with large differences in staining intensity from field to field (not shown). Similarly to α2, α3 subunit in wild-type CGC cultures appears to undergo developmental downregulation. However, the decrease in GAD colocalization was stronger for α3 than for α2 subunit, with more than threefold decrease of α3 expression at GAD-positive sites from DIV6 to -12 (Fig. 7, A and B). In contrast, GAD-positive puncta gradually became colocalized with α1 and α6 subunit clusters as development in vitro progressed. We quantified the percent of GAD puncta colocalized with each subunit’s clusters along a set length of randomly selected dendrites (Fig. 7B). These results taken together indicate that α3 subunit first and α2 subunit later are progressively replaced by α1 and α6 subunits at inhibitory synaptic sites in developing CGCs, supporting the critical role of these subunits in developmental regulation of synaptic efficacy seen with electrophysiological recordings. In addition, the number of synapses facing α1, α2, α3, and α6 subunits was always >100%, indicating that some α subunits must be colocalized at the same synaptic sites.

When the quantification of the percent of GAD-positive boutons was extended to CGCs from −/− mice, compensatory expression was seen for α2 and α3 subunits but not for α6 subunit (Fig. 7B). Colocalization of GAD terminals with α2 or α3 subunit staining was not significantly decreased in −/− CGCs between DIV-6-7 and DIV-8-9. However, at DIV-11-12, GAD/α3 subunit colocalization decreased in −/− CGCs, but GAD/α2 subunit colocalization did not and it was significantly higher than in +/+ CGCs. (Fig. 7B). In parallel experiments, we investigated the expression of β2/β3 as compared with α2 subunits in DIV12 CGCs from +/+ and −/− mice. The density of β2/β3 puncta per 16 μm dendritic length was higher for +/+ CGCs (7.0 ± 0.7) than for −/− CGCs (4.5 ± 0.8), reflecting the differing action of loreclezole on mIPSC decay between genotypes. The extent of cluster colocalization between β2/β3 and α2 subunits was significantly smaller for +/+ (39 ± 10%) than −/− CGCs (74 ± 10%) as an expected consequence of the decrease of α2 subunit expression in +/+ but not in −/− CGCs.

**DISCUSSION**

We followed developmental changes at inhibitory synapses made by GABAergic interneurons in cerebellar granule neurons in primary cultures from wild-type and α1 subunit knockout mice. The specific culture condition we used facilitates formation of functional inhibitory synapses allowing us to study them with whole cell voltage-clamp recordings and immunohistochemical staining. sIPSCs occur at high frequency as expected by the large number of GABAergic terminals seen with immunohistochemical methods and the high level of spontaneous activity of cerebellar GABAergic neurons (Farrant and Brickley 2003; Leao et al. 2000; Mellor et al. 2000). Our results indicate that the developmental changes of GABA<sub>A</sub>-mediated current kinetics and amplitude reported in cerebellar granule neurons in slices (Brickley et al. 1996; Tai et al. 1996; Vicini et al. 2001) can be observed in primary cultures of these neurons despite differences in synaptic connectivity. This provides a powerful model for investigation of the mechanisms underlying changes of inhibitory synaptic efficacy. An overview of the correlated immunohistochemical and electrophysiological findings is presented in Table 2.

As reported for IPSCs in CGCs in early postnatal cerebellar slices (Farrant and Brickley 2003), sIPSC and mIPSC decay is best fit by three exponential components. The fastest component of GABA responses can be seen with rapid agonist applications to excised patches but is generally lost when synaptic currents are small or distorted by dendritic filtering. The relatively large currents and high-quality voltage clamp that can be achieved with CGCs (Silver et al. 1992) allows effective resolution of all three components of the synaptic decay. If an exponential component were related to the presynaptic terminals of GABA responses can be seen with rapid agonist applications to excised patches but is generally lost when synaptic currents are small or distorted by dendritic filtering. The relatively large currents and high-quality voltage clamp that can be achieved with CGCs (Silver et al. 1992) allows effective resolution of all three components of the synaptic decay. If an exponential component were related to the presynaptic terminals of GABAergic interneurons in cerebellar granule neurons in primary cultures from wild-type and α1 subunit knockout mice.
the triple-exponential decay of mIPSCs was preserved during development, our data suggest rather that multiple decay components are characteristic of each of the distinct receptor subtypes as seen in responses obtained with ultra-rapid GABA applications from distinct recombinant receptors (Barberis et al. 2001), we observed that α1 deletion greatly attenuated the developmental acceleration of sIPSCs decay in cultures but did not completely prevent it. This suggests a role for additional factors in normal development or involvement of compensatory mechanisms in the α1 −/− cultures. To perform a more reliable assessment of the developmental changes at inhibitory synapses containing and lacking the α1 subunit, we focused on mIPSCs. The results of our analyses revealed that, in parallel to the decrease of the decay time of mIPSCs, there are also changes in mIPSCs amplitude and frequency of occurrence. A significant decrease of mIPSC amplitude was observed first in CGCs cultured from the −/− mice and only several days later in CGCs cultured from the +/+ animals. The weighted time constant declined, albeit to a different extent, in both the −/− and the +/+ groups. Consequently, the synaptic charge trans-

![Image](http://jn.physiology.org/)

**FIG. 7.** Synaptic localization of distinct α subunits in developing CGCs. A: high-magnification microphotographs illustrating neurons and dendrites from CGC cultures at DIV6 (top), DIV9 (middle), and DIV12 (bottom). Anti-α1, -α2, -α3, or -α6 immunostaining (middle) is matched to anti GAD immunostaining (top panels) and overlayed (bottom) to reveal synaptic localization of subunit clusters. Scale bar 5 µm. B: summary histogram illustrating the percent GAD-positive clusters colocalized with α1 (●), α2 (□), α3 (○) or α6 (●) subunits (% synapses) in CGCs from +/+ (top) and −/− (bottom) mice at 3 developmental age groups. *, P < 0.01 vs. α1; 196, P < 0.01 vs. α2; •, P < 0.05 vs. wild-type; +, P < 0.01 vs. DIV6-7.

### TABLE 2. Summary of subunit expression changes and corresponding functional changes and pharmacological sensitivities in IPSC parameters with development

<table>
<thead>
<tr>
<th>CGCs</th>
<th>α1</th>
<th>α2</th>
<th>α3</th>
<th>%Tw</th>
<th>Percentage Charge</th>
<th>Percentage zol</th>
<th>α6</th>
<th>Percentage fur</th>
<th>β2/β3</th>
<th>Percentage lor</th>
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<tr>
<td>DIV6-7</td>
<td>+/+</td>
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<td>92 ± 32</td>
</tr>
<tr>
<td></td>
<td>−/−</td>
<td>+</td>
<td>+</td>
<td>98</td>
<td>98</td>
<td>31 ± 21</td>
<td>+</td>
<td>8.8 ± 8.9</td>
<td>+</td>
<td>134 ± 19</td>
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<tr>
<td>DIV11-12</td>
<td>+/+</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>40</td>
<td>97 ± 26</td>
<td>↑</td>
<td>33 ± 4</td>
<td>↑</td>
<td>452 ± 85</td>
</tr>
<tr>
<td></td>
<td>−/−</td>
<td>—</td>
<td>⇆</td>
<td>⇆</td>
<td>78</td>
<td>19 ± 9*</td>
<td>↑</td>
<td>27 ± 4</td>
<td>⇆ 7*</td>
<td>168 ± 53*</td>
</tr>
</tbody>
</table>

* + or − indicate absence or presence of subunits; arrows (↑ ↓) indicate significant (P < 0.01) and not significant changes (↔) in percentage GAD-positive synapses containing a particular subunit relative to DIV6-7; *, significant (P < 0.05) changes compared to +/+ at the same DIV. To simplify presentation, we are only comparing changes of subunit expression with development; detail regarding relative proportions of each subunit at any given age can be found in the text. Values given are percentage change from mIPSC values at DIV6-7 in +/+ CGCs. Values given are percentage prolongation of τw of sIPSCs by 200 nM zolpidem (zol) (± SE) DIV8 instead of DIV6-7 data is presented here and for furosemide as DIV6 was our earliest data point in these experiments. Values are percentage prolongation of τw of sIPSCs by 10 µM loreclezole (lor) at DIV6-7 and 12-13 ± SE.

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fer dropped by a half in both the wild-type and the knock-out CGCs over a period of 2 days, suggesting that developmental changes in decay time and amplitude are related and might act to compensate each other. It is tempting to speculate that such compensatory action might underlie the lack of behavioral alterations in α1−/− mice (Kralic et al. 2002; Sur et al. 2001), although differences in charge transfer between genotypes were observed later in development. An additional consideration when evaluating developmental changes of mIPSCs amplitude are possible changes in the expression of potassium/chloride cotransporters (Payne et al. 2003; Staley and Smith 2001). These changes would not be apparent in our experimental conditions due to symmetrical chloride, yet they may have an important physiological role concomitant to the changes in GABA_A receptor subunit expression.

The probability of GABA release is also important for inhibitory synaptic efficacy and may change both with development and as a compensatory effect of the α1 subunit deletion. Developmental increase of mIPSC frequency was not correlated with an increase in inhibitory synaptic terminal density as seen with GAD staining. This indicates, most likely, that changes in the probability of release take place with development. Indeed, Studler et al. (2002) reported a significant enlargement of GABAergic terminals in developing CGC cultures. The delayed increase of mIPSCs frequency in α1 subunit −/− CGCs could be due to a smaller number of postsynaptic sites. The immunostaining data, however, show compensatory changes at postsynaptic sites with α2 and α3 subunits (see following text). Therefore the lower mIPSCs frequency seen in −/− CGCs is likely due to a compensatory alteration in release probability.

In previous immunocytochemical studies in CGC cultures, GABA_A receptor subunit clusters were described for α, β, and γ subunits (Balduzzi et al. 2002; Gao and Fritschy 1995; Studler et al. 2002). However, although individual well-defined clusters were sometime identified, staining was mostly dispersed with microclusters seen all over cell body and dendrites. In contrast, we observed well-defined clusters similar to those reported in hippocampal neurons in culture (Brunig et al. 2002; Christie et al. 2002; Levi et al. 2004). It is likely that the reason for this difference lies in the formation of functional synapses as seen with electrophysiological recordings. In addition, the use of insulin in the culture medium facilitates surface expression of GABA_A receptor subunits (Wan et al. 1997). Inhibition of sIPSCs by furosemide increased with development but was comparable between cells in the −/− and +/+ groups at all ages tested. Immunostaining with α6 subunit specific antibodies support the increase of α6 subunit expression at synapses. These data are consistent with the results in cerebellar slices suggesting that α6 subunits increasingly participate in inhibitory synaptic transmission in developing CGCs (Tia et al. 1996). We were not able to detect a significant staining with antibodies against the α5 subunit in contrast to the reported presence of this subunit in hippocampal cultures with the same antibody (Brunig et al. 2002). This result is consistent with the lack of α5 subunits mRNA in cerebellum (Laurie et al. 1992a,b).

The α4 subunit is mostly expressed in granule neurons of the hippocampus dentate gyrus, although there are reports indicating its presence in CGCs (Mascia et al. 2002; Pollit et al. 2003). Although we did not directly assess the expression of α4 subunit with immunocytochemical studies, we investigated its presence pharmacologically. The α4-selective allosteric modulator, bretazenil, (Knoflach et al. 1996) failed to change mIPSCs across ages or genotypes, indicating that α4 subunit is not expressed at synaptic sites. α4 and α6 subunits in combination with δ subunit are responsible for tonic activation of perisynaptic and extrasynaptic receptor in hippocampal and cerebellar granule neurons, respectively (Stell et al. 2003; Sundstrom-Poromaa et al. 2002; Wei et al. 2003). The whole cell current elicited by THIP, a superagonist at α4β3δ receptors, although with considerable potency and efficacy for containing receptors (Brown et al. 2002; Ebert et al. 1997), increased with development and was significantly higher in −/− CGCs. This suggests an increased expression of either α4β2δ or α6β2δ extrasynaptic receptors in the knock-outs.

Additional regulation of the efficacy of GABAergic synapses in CGCs with development may also be contributed by the isoforms of β or γ subunits. A similar action of flunitrazepam on mIPSCs at different DIV suggests that an increase of γ2 subunit may occur in parallel with the observed increase in benzodiazepine-insensitive α6 subunit at inhibitory synapses. In addition, greater prolongation of mIPSCs with development of +/+ but not −/− CGCs by loreclezole demonstrates a role for β2/3 subunits. Because the sensitivity to loreclezole of the α2/α3 subunit is similar to that of α1 subunit when coexpressed with β2 subunits (Wafford et al. 1994), our results indicate that the expression of the β2/3 subunits increased with development in wild-type but not in knock-out CGS. This is further supported by the greater density of β2/β3 subunit puncta in +/+ than in −/− CGCs. Studies of mIPSCs in neurons from β3−/− mice, suggested that α1-containing receptors were selectively retained by co-assembled β2 subunit (Ramadan et al. 2003). In addition, anatomical and biochemical data show preferential association of α2 with β3 subunit and of α1 with β2 subunit (Benke et al. 1994; Bollan et al. 2003; Fritschy et al. 1994). CGCs from α1−/− mice might thus be expected to have lower expression levels of functional β2 subunit-containing receptors.

In summary, the sequential expression of α3 followed by α2 and later by α1 subunits is the most likely regulator of inhibitory synaptic efficacy with development, although additional subunits may also be involved. Our results supplement previous findings in cerebellar slices and elucidate the role of distinct α subunits in regulation of functional efficacy of GABAergic networks.

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References


