Title
GABA(A) receptor-mediated miniature postsynaptic currents and alpha-subunit expression in developing cortical neurons.

Permalink
https://escholarship.org/uc/item/15f257tg

Journal
Journal of neurophysiology, 82(6)

ISSN
0022-3077

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Publication Date
1999-12-01

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Peer reviewed
INTRODUCTION

Inhibitory synaptic transmission in the mature cerebral cortex, mediated by GABA_ARs, plays a vital role in regulating normal cortical activity. Although GABAergic neurons are present and GABA_ARs are expressed in the neocortex of newborn rodents (Del Rio et al. 1992; Laurie et al. 1992), the inhibitory network is not fully mature at birth. Anatomic studies indicate a paucity of inhibitory synaptic contacts in animals <1 wk old (Blue and Parmavelas 1983; Miller 1986). In addition, the robust, short-latency GABA_A-R-mediated postsynaptic responses to stimulation of afferent fibers in the adult cortex are seen only rarely before the second postnatal week (Agmon and O'Dowd 1992; Burgard and Hablitz 1993; Luhmann and Prince 1991). More recent studies have identified symmetrical (presumed inhibitory) synapses at postnatal day 4 (P4) (De Felipe et al. 1997), and demonstrated that thalamic stimulation can evoke labile, asynchronous GABA_A-R-mediated synaptic currents in the mouse somatosensory cortex as early as P0 (Agmon et al. 1996). An immature chloride gradient appears to be responsible for the elevated reversal potential of the GABAergic synaptic currents observed in young cortical neurons (Owens et al. 1996). Age-related differences in GABA-evoked currents in acutely isolated cortical neurons suggest that alterations in the properties of the GABA_ARs may also contribute to maturation of GABAergic transmission (Oh et al. 1995). To address this question directly, however, it is necessary to examine the functional properties of the subpopulation of GABA_ARs that are specifically localized at synapses in developing cortical neurons.

The functional properties of ionotropic GABA_ARs can be influenced by a number of factors including receptor subunit composition, desensitization rates, phosphorylation state, as well as reuptake rates of the ligand (Angelotti and Macdonald 1993; Draguhn and Heinemann 1996; Galaretta and Hestrin 1997; Haas and Macdonald 1999; Jones and Westbrook 1995, 1997; Macdonald and Olsen 1994; Serafini et al. 1998; Verdoorn et al. 1990). Although some or all of these properties may be altered during development, a combination of biophysical and pharmacological studies suggest that changes in α-subunit composition contribute to early postnatal maturation of receptors mediating GABAergic currents in cerebellar (Mathews et al. 1994; Tia et al. 1996; Vicini 1999) and hippocampal neurons (Hollrigel and Soltesz 1997; Kapur and Macdonald 1999; Rovira and Ben-Ari 1993). These findings, in combination with previous reports of a large increase in α1 and a decrease in α5 GABA_A-R subunit expression in the developing rodent neocortex (Golshani et al. 1997; Laurie et al. 1992; Paysan et al. 1994), suggest that changes in expression of these α-subunits may contribute to maturational changes in the functional properties of GABA_ARs mediating inhibition in cortical neurons.

In this study whole cell recordings were used to examine the biophysical features of the GABAergic miniature postsynaptic currents (mPSCs), which reflect the functional properties of...
synaptically localized GABA<sub>A</sub>Rs, in cortical neurons developing in vivo and in dissociated cell culture. Having observed a developmental change in the kinetic properties of the GABAergic mPSCs, we sought to identify the molecular events that might underlie them. The sensitivity of the GABAergic mPSCs to modulation by zolpidem, a benzodiazepine type I (BZ1) agonist with high affinity for receptors containing α1- versus α5-subunits (Faure-Halley et al. 1993; Macdonald and Olsen 1994; Pritchett and Seeburg 1990), was used to probe for possible alterations in the relative contribution of these subunits in the functionally active synaptic GABA<sub>A</sub>Rs. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis was conducted to examine the developmental regulation of α-subunits (1, 2, 3, and 5) in RNA harvested from cortices or cultures at different ages. Finally, by combining whole cell recordings and gene expression in single neurons (Brooks-Kayal et al. 1998a,b; Eberwine et al. 1992; Lambez et al. 1992; O’Dowd and Smith 1996; Ruano et al. 1997), the correlation between α1 and α5 subunit expression and biophysical properties of the GABAergic mPSCs in individual cortical neurons was examined. Our data suggest that changes in α-subunit composition of synaptically localized GABA<sub>A</sub>Rs contribute to the maturation of inhibition in developing cortical neurons.

**METHODS**

**Tissue culture**

Primary neuronal cultures were prepared from mouse somatosensory cortex as previously described (Li et al. 1997; Massengill et al. 1997). Briefly, P0 mice (ICR, Harlan Sprague Dawley, San Diego, CA) were anesthetized by hypothermia before decapitation. The brain was removed, and pieces of the somatosensory cortex were dissected out and treated with papain (10 U/ml) for 30 min at 37°C. The tissue was mechanically dissociated, in neurobasal medium with B27 supplements (NMB + B27; Life Technologies, Gaithersburg, MD), using sterile glass micropipettes. The cells were plated onto poly-d-lysine–coated glass coverslips (Bellco Glass, Vineland, NJ) and maintained in 2% BSA-PBS containing 0.05% NaN<sub>3</sub> for 30 min on ice, washed in PBS and supplemented in PBS containing 0.02% saponin, 0.05% NaN<sub>3</sub> for 30 min on ice. Incubation with the primary antibody at a dilution of 1:1,000 (polyclonal anti-GABA antibody, Sigma) was carried out in 2% BSA-PBS containing 0.05% NaN<sub>3</sub> overnight at 4°C. Incubation with the secondary antibody at a dilution of 1:200 (Texas Red-conjugated anti-rabbit IgG, Vector Laboratories) was performed in 2% BSA-PBS containing 0.05% NaN<sub>3</sub> for 2 h at room temperature. Cultures were washed three times with 2% BSA-PBS after each incubation. To determine the mean incidence of GABA-positive neurons, cultures were viewed with Hoffman optics to count the total number of neurons in randomly chosen fields of view. Fluorescent illumination of the same fields was used to count the number of labeled neurons. The mean percentage of GABA-positive neurons was determined from counts obtained from five fields on seven or more coverslips at each developmental stage.

**Electrophysiology (cultured neurons)**

Whole cell recordings were made from cultured somatosensory cortical neurons [3–28 days in vitro (DIV)] using unpolished pipettes with an open tip resistance of 1–3 MΩ. The internal pipette solution contained (in mM) 120 KCl, 20 NaCl, 2 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 1 EGTA, and 10 HEPES, pH 7.2. Cultured neurons were bathed in an external solution containing (in mM) 140 NaCl, 3 KCl, 4 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, and 5 HEPES, pH 7.2. The following drugs were added to the external solution and bath applied in various combinations as required: 1 µM tetrodotoxin citrate (TTX), 5 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 50 µM D(-)-2-amino-5-phosphonopentanoic acid (APV), and 2 µM D(-)-bicuculline methiodochrome (BMC) and 3–3,000 nM N,N,6-trimethyl-2-(4-methylphenyl)-imidazo[1,2-a] pyridine-3-acetamide (Zolpidem) (all from Research Biochemicals). Data were acquired using a patch-clamp amplifier (List EPC-7; Axopatch 1-D), a D-A board (Labmaster or DigiData 1200A; Axon Instruments), and pClamp6 (Axon Instruments) or SCAN (Courtesy of Dr. J. Dempster, Strathclyde Electrophysiology Software, Strathclyde University, Strathclyde, UK) software running on a Dell 386 or Pentium PC. The signal was filtered at 2.5–5 kHz and digitized at 1–10 kHz.

**DATA ANALYSIS**  
**mPSC frequency** was determined in each neuron from 30, 1-s current traces, filtered at 2.5 kHz, and digitized at 1 kHz using pCLAMP software. Individual events were counted when the amplitude was >20 pA (4-fold greater than the average RMS noise level of 5 pA). **mPSC biophysical properties** were determined from records filtered at 2.5–5 kHz and digitized at 5–10 kHz using SCAN. The mean amplitude and 10–90% rise time were determined by averaging the values obtained from ≥50 single events in each neuron. Decay kinetics were evaluated by two different measures. The T50% value was defined as the time required for the ensemble average mPSC from each neuron to decay to 50% of the peak amplitude (Hajos and Mody 1997). Second, the decay time constant for each neuron was determined by fitting a single exponential to the falling phase of the ensemble average mPSC.

**Electrophysiology (acute slice preparation)**

Neonatal mice age 8 and 20–23 days postnatal were used for electrophysiological recordings in an acute slice preparation as previously described (Hollrigel and Soltesz 1997; Hollrigel et al. 1998). Briefly, mice were anesthetized by halothane inhalation before killing by decapitation. The brain was removed and placed in ice-cold oxygenated (95% O<sub>2</sub>-5% CO<sub>2</sub>) artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 2.5 KCl, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 1.25 NaHPO<sub>4</sub>, and 10 mM glucose. Coronal brain slices processed with a vibratome tissue sectioner (Lancer Series 1000), were equilibrated for 1 h in ACSF at room temperature before recording. Individual slices were transferred to a submersion recording chamber perfused with ACSF bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub> containing 10 µM APV, 5 µM CNQX, and 1 µM TTX. The internal pipette solution contained (in mM) 120 KCl, 20 NaCl, 2 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 1 EGTA, and 10 HEPES, pH 7.2. Blind whole cell recordings were obtained as described previously (Blanton et al. 1989) using an Axopatch-200A amplifier. All recordings, from neurons in layers V and VI, were performed at room temperature.

**RT-PCR**

Mice at P7, P14, P21, and P28 were anesthetized by halothane inhalation before decapitation. Brains were removed and the somatosensory cortex dissected free from the surrounding tissue. Total RNA was isolated from acutely dissociated cortical tissue and cultures at 7, 14, 21, and 28 DIV, by a single step method (Chomczynski and Sacchi 1987). First strand cDNA was synthesized by reverse transcription of 100 ng total RNA as described (O’Dowd et al. 1995). **RT-PCR amplification of α1, α2, α3, α5:** PCR products were
amplified in separate single round RT-PCR reactions, using primer pairs specific for each of the four distinct GABA<sub>A</sub>R α-subunits (Table 1). PCR parameters for amplification were as follows: 1 cycle at 94°C for 1 min, 20 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min, and a final elongation cycle at 72°C for 6 min. The identity of each of the PCR products was confirmed by sequencing. *Multiplex RT-PCR amplification of α1 and α5*: Co-amplification of the products for α1 and α5 was accomplished by combining the individual primer sets in a single round RT-PCR reaction using the same amplification conditions stated above.

In both the single and multiplex RT-PCR reactions, forward primers were radiolabeled by a T<sup>4</sup> DNA kinase reaction (Promega) with <sup>32</sup>P-ATP (NEN-DuPont), and ~5 × 10<sup>4</sup> cpm of <sup>32</sup>P-labeled primer was added to the PCR. The radiolabeled PCR products were separated by electrophoresis on a nondenaturing 8% polyacrylamide gel. Quantitative analysis was performed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

**Single-cell multiplex RT-PCR**

Analysis of GABA<sub>A</sub> receptor α-subunit mRNA expression was performed on RNA harvested from single cells as described (Massengill et al. 1997). Briefly, after whole cell electrophysiology, mild suction was used to aspirate the contents of the cell into the tip of the recording electrode that was then expelled into a tube containing reverse transcription buffer. First-strand cDNA synthesis was initiated by the addition of 100 units of Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies), and the reaction was allowed to proceed for 1 h at 37°C. After termination of the reaction, the resulting cDNAs for the α1 and the α5-subunit of the GABA<sub>A</sub>R were co-amplified in a multiplex PCR reaction, using two rounds of amplification, with distinct sets of nested primers specific for α1 and α5 (Table 1). First round PCR parameters for co-amplification of α1 and α5 were as follows: 1 cycle at 94°C for 1 min, 40 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min, and a final elongation cycle at 72°C for 6 min. The first round products were diluted at 1:1,000 and amplified in a second-round under the following conditions: 1 cycle at 94°C for 1 min, 40 cycles at 94°C for 30 s, 54°C for 1 min, and 72°C for 1.5 min, and a final elongation cycle at 72°C for 6 min. PCR products were labeled by inclusion of <sup>32</sup>P-labeled primer, separated by gel electrophoresis, and analyzed on a phosphorimager.

**Results**

To determine whether there are maturation changes in the properties of GABA<sub>A</sub>R mediating synaptic transmission in the neocortex, GABAergic mPSCs were examined in cortical neurons during early postnatal development, both in vivo and in vitro. A previous study from our lab demonstrated that GABA<sub>A</sub>R-mediated currents could be recorded from cortical neurons in brain slices as early as the day of birth (Agmon et al. 1996). Here we demonstrate that GABA<sub>A</sub>Rs also mediate functional transmission between cortical neurons, harvested from P0 mouse somatosensory cortices, grown in dissociated cell culture. Fast, action potential (AP)–independent postsynaptic potentials and currents (mPSPs and mPSCs) were observed in the majority of cultured neurons examined (Fig. 1A). These were mediated by GABA<sub>A</sub>Rs based on the demonstration that the mPSPs and mPSCs were reversibly blocked by BIC, a GABA<sub>A</sub>R antagonist, but were not affected by the glutamate receptor antagonists CNQX and APV (Fig. 1A). In addition, the mPSCs exhibited a reversal potential near the calculated chloride equilibrium potential (E<sub>Cl</sub> = −2 mV) as expected for currents mediated by activation of GABA<sub>A</sub>Rs (Fig. 1B).

**Frequency of GABAergic mPSCs in cortical neurons developing in vitro and in vivo**

GABAergic mPSCs could be recorded from some neurons as early as the third day in culture (3 DIV). However, because of the low incidence of observing neurons with these currents at 3–4 DIV, quantitative analysis of GABAergic mPSCs in the first postnatal week was limited to cells between 5 and 7 DIV. In this age range, GABAergic mPSCs were observed from ~75% of the neurons examined (Fig. 2A). From the second week on, GABAergic mPSCs were present in nearly all of the neurons examined (Fig. 2A). A progressive change in the average frequency of GABAergic mPSCs was observed, ranging from <1 Hz at 5–7 DIV up to 8 Hz at 4 wk (Fig. 2, B and C). A large increase in the frequency of GABAergic mPSCs also occurred in cortical neurons developing in vivo. Recordings from neurons in slices prepared at P8 revealed an average mPSC frequency of 0.15 ± 0.04 Hz (mean ± SE, n = 9) as compared with 2.7 ± 1.01 Hz (mean ± SE, n = 5) in neurons examined in slices made from animals at P20–23.

To determine whether developmental changes in the number of GABAergic neurons might contribute to the increase observed in the mPSC frequency, staining with anti-GABA an-
tibodies was used to assess the percentage of GABA-positive neurons at different times in culture (Fig. 3, A and B). The average percentage of GABA-positive neurons (15%) did not change significantly between 1 and 4 wk in vitro (Fig. 3C) demonstrating that the developmental increase in mPSC frequency is not a consequence of a change in the number of GABAergic neurons.

**Biophysical properties of GABAergic mPSCs in cortical neurons developing in vitro and in vivo**

To address the question of whether there may be alterations in the functional properties of synaptic GABA\textsubscript{A}Rs, the biophysical properties of GABAergic mPSCs were assessed through analysis of 50–1,000 individual mPSCs recorded from single neurons between 1 and 4 wk in culture. Qualitative comparison of currents observed in old versus young neurons suggested that the currents decayed more rapidly in the older neurons (Fig. 4A). Two independent measures were employed to quantitatively evaluate this potential change in decay kinetics. First, a decay time constant was determined by fitting an exponential function to the falling phase of the ensemble average mPSC in each neuron. Although the falling phase of some individual mPSCs could be best fit by the sum of more than one exponential, the ensemble average in the vast majority of neurons at all ages examined were adequately fit by a single exponential (Fig. 4A). Second, the time for the mPSC to decay to half-amplitude (T50%) was determined from the ensemble average mPSC in each neuron. Examination of both the mean decay time constant and the T50% values as a function of age revealed a two- to threefold decrease during the first 4 wk in culture from $>30$ ms during the first 2 wk, to $\sim 15$ ms during the 4th week (Fig. 4B and C). The mean mPSC amplitude and rise time were determined by averaging the values obtained from 50 or more single mPSCs in each neuron. In contrast to the change in decay kinetics, there was no significant change in either the mean amplitude or rise time of the mPSCs during the first 4 wk in culture (Fig. 4D and E). The developmental increase in the rate of decay of the GABAergic mPSCs in the absence of alterations in amplitude supports the hypothesis that regulation of channel properties, rather than simply an age-dependent alteration in electronic filtering, underlies this change.

Analysis of GABAergic mPSCs from neurons in acute slices prepared from animals at P8 and P20–23 revealed ensemble average mPSCs with more rapid decay kinetics in the older versus younger neurons (Fig. 4F). The mean mPSC decay time constant was significantly faster (3.5-fold) at P20–P23 when compared with P8 (Fig. 4G). Similar to the results in vitro, there was no significant change in the amplitude of the GABAergic mPSCs during this developmental period in vivo (Fig. 4H). These data suggest that changes in functional properties of synaptically localized GABA\textsubscript{A}Rs contribute to development of inhibition in the rodent neocortex.

**Zolpidem sensitivity**

The developmental increase in \(\alpha 1\) and decrease in \(\alpha 5\) GABA\textsubscript{A}R subunit expression in the rodent neocortex (Gol-
from 12 neurons at 4 wk revealed a significant increase in amplitude (23 ± 8%; P < 0.01, paired Student’s t-test) and decay time constant (34 ± 10%, P < 0.01, paired Student’s t-test) following exposure to 300 nM zolpidem. A less pronounced, but still significant, change was observed in the amplitude and decay time constant of neurons at 7 DIV (Fig. 5, B and D). Zolpidem (300 nM) enhanced the mPSC amplitude by 14 ± 6% (P < 0.05, paired Student’s t-test) and decay time constant by 21 ± 5% (P < 0.01, paired Student’s t-test) in the 16 cells examined at 5–7 DIV.

FIG. 3. Subpopulation of GABA-positive neurons in cortical cultures. Cultures were fixed, stained, and visualized by indirect immunofluorescence using an anti-GABA antibody. A: photomicrograph of a typical culture fixed at 7 DIV and viewed with Hoffman modulation contrast optics. B: epifluorescent illumination identifies 2/9 of the neurons as GABA-positive neurons. Scale bar, 20 μm. C: quantitative analysis of the percentage of GABA-positive neurons during the 1st 4 wk in vitro reveals no significant change during this time period. Bars indicate SE.
To further explore differences in the sensitivity of neurons to zolpidem, we plotted the percent change in decay kinetics and amplitude as a function of zolpidem concentration in neurons at 5–7 versus 22–28 DIV (Fig. 5, E and F). The mPSCs recorded from neurons in both age groups show a dose-dependent increase in their decay time constant. However, the shift to the left in the curve generated from the older neurons demonstrates a developmental increase in sensitivity to zolpidem with a significant difference observed at 30 nM zolpidem \((P, 0.05, \text{unpaired Student's } t\text{-test; Fig. 5E})\). We were not able to determine whether there was an age-related difference in the amplitude enhancement due to the relatively large variability observed in the neurons at both 1 and 4 wk (Fig. 5F).

**Culture**

A

28 DIV

\(\tau = 16.5 \text{ ms} \quad \text{T50\%} = 15.0 \text{ ms}\)

7 DIV

\(\tau = 42.6 \text{ ms} \quad \text{T50\%} = 40.0 \text{ ms}\)

B

Age in Culture (DIV)

Decay time constant (ms)

\(\begin{array}{cccc}
5-7 & 8-14 & 15-21 & 22-28 \\
\text{(31)} & \text{(30)} & \text{(50)} & \text{(30)}
\end{array}\)

C

Age in Culture (DIV)

T50\% (ms)

\(\begin{array}{cccc}
5-7 & 8-14 & 15-21 & 22-28 \\
5 & 10 & 15 & 20
\end{array}\)

D

Age in Culture (DIV)

Amplitude (pA)

\(\begin{array}{cccc}
5-7 & 8-14 & 15-21 & 22-28 \\
10 & 20 & 30 & 40
\end{array}\)

E

Age in Culture (DIV)

10–90\% rise time (ms)

\(\begin{array}{cccc}
5-7 & 8-14 & 15-21 & 22-28 \\
1 & 2 & 3 & 4
\end{array}\)

**Slice**

F

P23, \(\tau = 11.8 \text{ ms}\)

P8, \(\tau = 30.7 \text{ ms}\)

G

Age in Culture (DIV)

Decay time constant (ms)

\(\begin{array}{cccc}
5-7 & 8-14 & 15-21 & 22-28 \\
5 & 10 & 15 & 20
\end{array}\)

H

Age in Culture (DIV)

Amplitude (pA)

\(\begin{array}{cccc}
5-7 & 8-14 & 15-21 & 22-28 \\
10 & 20 & 30 & 40
\end{array}\)

\(\text{FIG. 4. Increase in rate of decay of GABAergic mPSCs during development in vitro and in vivo. A: ensemble average mPSCs (solid lines) recorded from 2 representative neurons at 7 and 28 DIV. Traces were normalized to a unitary amplitude. A single exponential (dashed lines) with the indicated decay time constant (}\tau\text{) is fit to the data. B: the mean mPSC decay time constant decreases significantly as a function of increasing age in culture (ANOVA; } P < 0.0001\text{). C: in the same population of neurons, the mean T50\% also decreases significantly as a function of age in culture (ANOVA; } P < 0.0001\text{). D and E: no significant change was observed in the amplitude or the 10–90\% rise time in these neurons. F: ensemble average mPSCs (solid lines) recorded from 2 representative neurons in slices prepared from the somatosensory cortices of mice at 8 and 23 days postnatal. Traces were normalized to a unitary amplitude. A single exponential (dashed lines) with the indicated decay time constant (}\tau\text{) is fit to the data. G: the mean mPSC decay time constant is significantly slower in the neurons examined in slices at postnatal day 8 (P8) compared with P20–23 (P < 0.01, Student’s } t\text{-test). H: the amplitude for this same population of neurons was similar at the 2 different ages. Error bars indicate SE, and the number of neurons is in parentheses.}\)
eightfold increase in the expression of α1 between 7 and 28 DIV (P < 0.01, paired Student’s t-test; Fig. 6C). A decrease in α5 expression occurred over this same time period (P < 0.02, paired Student’s t-test; Fig. 6F). Using identical amplification conditions and 100 ng total RNA, a similar developmental change was observed in the expression of the α1- and α5-subunits in RNA prepared from the somatosensory cortices of mice from P7–P28 (Fig. 6, B, D, and F). RT-PCR analysis of two additional subunits, α2 and α3, demonstrated that, although these were expressed throughout the developmental period examined, no significant change in expression was consistently observed either in vivo or in vitro (data not shown).

In these initial experiments, RNA was prepared from both somatosensory cortical tissue and dissociated cell cultures that are composed of a mixed population of cells including neurons and nonneuronal cells. To determine whether the changes in α1- and α5-subunit expression were representative of changes occurring in the neurons, we used a single cell multiplex RT-PCR approach to assess the expression of both subunits in single neurons at different ages in culture. After two rounds of amplification using nested primers, PCR products representing α1 and/or α5 were amplified from the majority of neurons between 7 and 28 DIV (70/104), in which GABAergic mPSCs were recorded. Regardless of developmental age, both α1 and α5 products were amplified from most neurons (Fig. 7A).

However, consistent with our analysis of RNA from whole culture, the frequency of encountering a single neuron in which only α5 was detected decreased with age in culture, whereas the frequency of encountering neurons in which only α1 was detected increased with age in culture (Fig. 7B). These data suggest that changes in the relative expression of α1- and α5-subunits contribute to changes in the GABAergic synaptic currents in developing cortical neurons.

α-Subunit expression in single neurons is correlated with mPSC decay kinetics

To examine the relationship between α-subunit expression and the rate of decay of mPSCs in individual neurons, cells were grouped into three categories based on the PCR products amplified (i.e., α5 only, α1 + α5, and α1 only). A representative GABAergic mPSC from single neurons in each of the PCR categories is shown in Fig. 8A. Cell 1, in which only α5 was amplified had a relatively slow decay time constant. Cell 2 expressing both α1 and α5 had an intermediate rate of decay, whereas cell 3, in which only α1 was amplified, had the fastest rate of decay. Analysis of all the neurons in which at least one PCR product was amplified revealed that the decay time constant in cells in which only α5 mRNA was detected was significantly slower than in those cells in which only α1 mRNA was detected (Fig. 8B). This was not simply an age-
dependent phenomenon because a similar correlation was observed when analysis was restricted to neurons in the third week in culture (Fig. 8C). In contrast, there was no correlation between a-subunit expression and mPSC amplitude (data not shown). These data support the hypothesis that an increase in the ratio of a1:a5-subunit expression contributes to an increased rate of decay in the GABAergic mPSCs.

**DISCUSSION**

This study documents changes in the kinetic properties of GABAergic mPSCs in mouse cortical neurons during the first postnatal month, both in vivo and in vitro, indicating that functional alterations in active, synaptically localized GABA\(_A\)Rs contribute to development of inhibition in the rodent neocortex. Sensitivity of young cultured neurons to low concentrations of zolpidem and the expression of GABA\(_A\)R a1-subunits, in RNA harvested from cortical tissue and single neurons in the first week, suggest that a1-subunits contribute to receptors mediating the mPSCs as early as the first postnatal week. However, developmental increases in zolpidem sensitivity, mPSC rate of decay, and the ratio of GABA\(_A\)R a1 to a5-subunit mRNA, suggest that an increase in the proportion of synaptic GABA\(_A\)Rs containing a1-subunits contributes to the maturation of GABAergic transmission in mouse cortical neurons.

**Maturation in the functional properties of GABA\(_A\)Rs mediating synaptic transmission in cortical neurons**

Previous studies have provided evidence of developmental changes in the functional properties of synaptically localized GABA\(_A\)Rs that are likely to contribute to development of inhibition in the mammalian CNS. In cerebellar granule cells, the fast exponential component of the decay phase of spontaneous inhibitory postsynaptic currents (IPSCs) becomes more...
prominent, and the IPSC amplitude decreases during the early postnatal period (Tia et al. 1996). A dramatic increase in the mPSC frequency and a twofold decrease in the decay time of the ensemble average mIPSCs, in the absence of a change in mIPSC amplitude, has been observed in dentate granule cells in rats between P0–P14 and adult (Hollrigel and Soltesz 1997). In both of these cases, a temporal correlation between eye opening/exploration of the environment (P14) with the changes in properties of the GABAergic currents has led to the suggestion that alterations in sensory stimulation may trigger, directly or indirectly, the changes in GABA<sub>R</sub> function. Our results from cortical neurons illustrate changes in mPSC frequency and kinetics that are similar to those reported in dentate granule cells, suggesting that the factors influencing development of these properties might be similar in neocortex and hippocampus. However, the observation that the temporal sequence and direction of the changes in GABAergic mIPSCs in cortical neurons developing in dissociated cell culture are similar to

FIG. 7. Developmental change in the frequency of single neurons in which only α<sub>1</sub>- or only α<sub>5</sub>-subunit mRNA was detected. Products unique for α<sub>1</sub>- and/or α<sub>5</sub>-subunit mRNA were amplified following reverse transcription and 2 rounds of multiplex PCR amplification using 2 nested primer sets specific for the different subunits. A: autoradiograms of the products amplified from RNA harvested from cultures at 14 DIV (R) and from 3 representative neurons at 7 and 28 DIV. Both subunits are amplified in the whole RNA sample as well as 2 of the single neurons at 7 and 28 DIV. In contrast, only α<sub>5</sub> was amplified in cell 1 at 7 DIV, and only α<sub>1</sub> was amplified from cell 6 at 28 DIV. Experiments were accepted for analysis only when media controls (mc), assayed every 3–5 cells, were negative. B: the majority of neurons sampled in each age group expressed both α<sub>1</sub> and α<sub>5</sub> mRNA. However, the frequency of cells in which only α<sub>5</sub> was amplified was highest between 7 and 14 DIV, whereas the frequency of cells in which only α<sub>1</sub> was amplified was highest at 22–28 DIV. Numbers in parentheses indicate number of neurons sampled at each age in which at least 1 PCR product was amplified.

FIG. 8. Correlation between expression of α-subunit expression and mPSC decay time constant in single neurons. A: autoradiogram of the PCR products amplified from RNA (R) harvested from a whole culture and from 3 individual neurons in RT-multiplex PCR reactions. Media control (mc). The averaged mPSCs (solid lines) for these same cells are shown to the right, along with their single exponential fits (dashed lines) with the indicated decay time constant (τ). Traces have been normalized to the same peak current amplitude. Cell 1, in which only α<sub>5</sub> was detected, had the slowest decay time constant, whereas cell 3, in which only α<sub>1</sub> was amplified, had the fastest decay time constant. B: population analysis of all the neurons between 7 and 28 DIV revealed that the average decay time constant in neurons in which α<sub>5</sub> only was detected was significantly longer than that seen in α<sub>1</sub> + α<sub>5</sub> (ANOVA; P < 0.01, Fishers PLSD) and in α<sub>1</sub> neurons (ANOVA; P < 0.001, Fishers PLSD). C: analysis of neurons between 15 and 21 days revealed a similar correlation between decay time constant and α-subunit expression group. Error bars indicate SE, and numbers in parentheses indicate number of neurons.
Evidence that changes in GABA_A receptors during the first postnatal week.

Changes in inhibitory synaptic currents in various regions of the brain have been shown to play an important role in developmental changes. For example, studies in the cerebellum have demonstrated that changes in GABAergic synapses during ontogeny are associated with alterations in sensory input and cell autonomous gene expression. In this study, we present evidence that changes in GABA_A Rs during the first postnatal week provide evidence of these changes.

Zolpidem has been a useful tool in gathering information regarding the subunit composition of native receptors. Sensitivity to modulation by low concentrations of zolpidem suggest that α1-subunit containing receptors contribute to the GABA-evoked currents or IPSCs in a variety of cells including dentate granule neurons (De Koninck and Mody 1994; Soltész and Mody 1994), cultured hippocampal neurons (Schonrock and Bormann 1993), cerebellar Purkinje cells (Itier et al. 1996), and cortical neurons (Gibbs et al. 1996; Perrais and Ropert 1999). A reversible increase in the mPSC amplitude and a slowing in the decay time, induced by bath exposure to zolpidem (300 nM) in the population of neurons examined between 5 and 7 DIV, demonstrate that BZ1-sensitive receptors contribute to the GABA_A Rs mediating synaptic currents in the first week. In addition, single-cell RT-mPCR analysis revealed that both α1- and α5-subunits could be amplified from the majority of neuronal subpopulations, even the youngest age group. These findings suggest that α1-subunits contribute to the formation of functional receptors mediating GABAergic mPSCs as early as the first postnatal week.

Evidence that changes in GABA_A α-subunit expression contribute to the development of GABAergic synaptic transmission in cortical neurons

An increase in the expression of the α6 GABA_A subunit has been shown to play an important role in developmental changes in inhibitory synaptic currents in cerebellar granule neurons (Tia et al. 1996; Zhu et al. 1996). Studies in hippocampal neurons indicate an increased potency of zolpidem in modulating GABA_A-mediated currents during maturation, consistent with the hypothesis that an increase in α1-subunit-containing receptors contributes to the developmental changes observed in the GABAergic synaptic currents (Hollrigel and Soltész 1997; Kapur and Macdonald 1999; Rovira and Ben-Ari 1993). In this study, we present evidence supporting the hypothesis that developmental changes in α-subunit expression also contribute to alterations in GABAergic transmission in cortical neurons. First, an increase in the sensitivity of mPSCs to bath application of zolpidem during development in vivo is consistent with an increase in the contribution of α1-subunits to GABA_A Rs mediating synaptic transmission. Although we did not investigate the pharmacological properties of neurons developing in the animal, a recent study demonstrates that zolpidem, at room temperature, enhances both the amplitude (38%) and the duration (63%) of the mPSC recorded from layer V pyramidal neurons located in the visual cortex of rat (Perrais and Ropert 1999). These latter studies were done in slices obtained from animals at P15–25, and the magnitude of the modulation by zolpidem is consistent with that seen in our older age group. Second, the striking increase in the level of α1 mRNA expression suggests that an increase in the number of α1-subunit-containing receptors contributes to the increase in mPSC frequency that occurs during the same developmental period. Finally, our single-cell RT-PCR analysis supports the hypothesis that a relative increase in expression of α1:α5 is important in the functional maturation of the decay kinetics of synaptic GABA_A Rs.

In addition to α1 and α5, α2–α4 are also expressed in the developing rodent neocortex. A recent report demonstrating that the majority of single pyramidal neurons in slices of the visual cortex expressed more than two, and as many as five (α1–α5) GABA_A subunits (Ruano et al. 1997), raises the question of the role of other α-subunits in maturational changes in the GABAergic mPSCs. Developing cultured cortical neurons showed little change in expression of α2- and α3-subunits, making it unlikely that these subunits contribute to the developmental changes in the kinetic and pharmacological properties of the mPSCs we report. However, changes in α4-subunits, that were not monitored in this study, may contribute to maturation of the GABAergic mPSCs based on the report of an increase in α4 mRNA expression during early postnatal development in the mouse somatosensory cortex (Golshani et al. 1997).

Factors important in regulating the development of GABAergic synaptic transmission

Cortical function is critically dependent on the normal maturation of the intrinsic membrane properties of its component neurons and the pattern of excitatory and inhibitory connections formed between the individual neurons. In the present study, comparison of the development of cortical neurons in dissociated cell culture with those developing in vivo resulted in identification of functional and molecular properties of the GABAergic synaptic transmission system that can develop in the absence of the normal pattern of afferent and efferent connections. However, it is clear from a number of studies that patterns of innervation (Payan et al. 1997) and receptor activation (Poulter et al. 1997) can regulate GABA_A Rs mediating synaptic transmission in cortical neurons. Thus continued exploration of the functional and molecular properties of GABA_A Rs mediating synaptic transmission in developing cortical neurons, under a variety of conditions, will be important in identifying environmental and genetic factors that can influence GABAergic synaptic transmission in cortical neurons.

The authors thank A. Agmon for comments on the manuscript and B. Nicolas for expert technical assistance.


