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Authors

Dunning, D D
Hoover, C L
Soltesz, I
et al.

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$GABA_A$ Receptor–Mediated Miniature Postsynaptic Currents and α-Subunit Expression in Developing Cortical Neurons

D. D. DUNNING,[1] C. L. HOOVER,[1] I. SOLTESZ,[1] M. A. SMITH,[1] AND D. K. O'DOWD[1,2]
[1]*Department of Anatomy and Neurobiology and* [2]*Developmental and Cell Biology, University of California, Irvine, California 92697-1280*

Dunning, D. D., C. L. Hoover, I. Soltesz, M. A. Smith, and D. K. O'Dowd. $GABA_A$ receptor mediated miniature postsynaptic currents and α-subunit expression in developing cortical neurons. *J. Neurophysiol*. 82: 3286–3297, 1999. Previous studies have described maturational changes in GABAergic inhibitory synaptic transmission in the rodent somatosensory cortex during the early postnatal period. To determine whether alterations in the functional properties of synaptically localized $GABA_A$ receptors ($GABA_A$Rs) contribute to development of inhibitory transmission, we used the whole cell recording technique to examine GABAergic miniature postsynaptic currents (mPSCs) in developing cortical neurons. Neurons harvested from somatosensory cortices of newborn mice showed a progressive, eightfold increase in GABAergic mPSC frequency during the first 4 wk of development in dissociated cell culture. A twofold decrease in the decay time of the GABAergic mPSCs, between 1 and 4 wk, demonstrates a functional change in the properties of $GABA_A$Rs mediating synaptic transmission in cortical neurons during development in culture. A similar maturational profile observed in GABAergic mPSC frequency and decay time in cortical neurons developing in vivo (assessed in slices), suggests that these changes in synaptically localized $GABA_A$Rs contribute to development of inhibition in the rodent neocortex. Pharmacological and reverse transcription-polymerase chain reaction (RT-PCR) studies were conducted to determine whether changes in subunit expression might contribute to the observed developmental alterations in synaptic $GABA_A$Rs. Zolpidem (300 nM), a subunit-selective benzodiazepine agonist with high affinity for α1-subunits, caused a reversible slowing of the mPSC decay kinetics in cultured cortical neurons. Development was characterized by an increase in the potency of zolpidem in modulating the mPSC decay, suggesting a maturational increase in percentage of functionally active $GABA_A$Rs containing α1 subunits. The relative expression of α1 versus α5 $GABA_A$R subunit mRNA in cortical tissue, both in vivo and in vitro, also increased during this same period. Furthermore, single-cell RT-multiplex PCR analysis revealed more rapidly decaying mPSCs in individual neurons in which α1 versus α5 mRNA was amplified. Together these data suggest that changes in α-subunit composition of $GABA_A$Rs contribute to the maturation of GABAergic mPSCs mediating inhibition in developing cortical neurons.

INTRODUCTION

Inhibitory synaptic transmission in the mature cerebral cortex, mediated by $GABA_A$Rs, plays a vital role in regulating normal cortical activity. Although GABAergic neurons are present and $GABA_A$Rs 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 Parnavelas 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 suggests that alterations in the properties of the $GABA_A$Rs 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 $GABA_A$Rs that are specifically localized at synapses in developing cortical neurons.

The functional properties of ionotropic $GABA_A$Rs 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; Galarreta 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 $GABA_A$Rs 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

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synaptically localized $GABA_A$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 $GABA_A$Rs. Semiquantitative 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; Lambolez 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 $GABA_A$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 a 5% CO_2 incubator at 37°C overnight. The following day, coverslips were transferred to dishes containing confluent nonneuronal feeder cultures in NBM + B27 or fed with media conditioned by feeder cultures. Coverslips were subsequently transferred to new feeder cultures or supplemented with fresh conditioned medium every 3 to 5 days, and were maintained in this manner for up to 4 wk.

Immunocytochemistry

Neuronal cultures were rinsed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde-PBS for 1 h on ice, washed in PBS, and permeabilized in PBS containing 0.02% saponin, 0.05% NaN_3 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_3 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_3 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 $MgCl_2$, 0.1 $CaCl_2$, 1 EGTA, and 10 HEPES, pH 7.2. Cultured neurons were bathed in an external solution containing (in mM) 140 NaCl, 3 KCl, 4 $MgCl_2$, 1 $CaCl_2$, 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 (−)-bicuculline methochloride (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_2-5% CO_2) artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, and 10 D(+)-glucose. Coronal brain slices prepared 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_2-5% CO_2 containing 10 μM APV, 5 μM CNQX, and 1 μM TTX. The internal pipette solution contained (in mM) 120 KCl, 20 NaCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 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 cultured neurons 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

TABLE 1. *Primers used in RT-PCR*

Subunit	Primer Set	Primer Sequence	Nucleotide Position	Fragment Size
α1*	Outer f1:	5′-CCA GTT TCA GAC CAC GAT ATG GAG-3′	380-403	285 bp
	Outer r1:	5′-GAA GTC TTC CAA GTG CAT TGG G-3′	664-643	
	Inner f2:	5′-TTC AAA GGA CCC ATG ACA GTG C-3′	455-476	150 bp
	Inner r2:	5′-AGT GCC ATC CTC TGT GAT ACG C-3′	604-583	
α2*	Outer f1:	5′-CGA ATC CAG GAT GAT GGA ACA TTG-3′	481-504	147 bp
	Outer r1:	5′-TGT GAC TTC TGA GGT TGT GTA AGC G-3′	627-603	
α3*	Outer f1:	5′-CGA CAA GAA CCT GGG GAC TTT G-3′	277-298	329 bp
	Outer r1:	5′-GCC AGA AGA TTG TTC AGT GGA AGG-3′	605-582	
α5†	Outer f1:	5′-GCG AAC AGA CAT CTA TGT TAC CAG C-3′	544-568	229 bp
	Outer r1:	5′-CTT GTT GGG TGT CGT CAT GTT G-3′	772-751	
	Inner f2:	5′-TTC CGT CAA AGC TGG AAA GAT G-3′	617-638	113 bp
	Inner r2:	5′-TTG TGG AAG AAT GTG TCT GGG GTC-3′	729-706	

* Mouse; Genbank accession no. M86566; Wang et al. (1992).
† Rat; Genbank accession no. X51992; Malherbe et al. (1990).

amplified in separate single round RT-PCR reactions, using primer pairs specific for each of the four distinct $GABA_AR$ 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 T4 DNA kinase reaction (Promega) with ^{32}P-ATP (NEN-DuPont), and $\sim 5 \times 10^5$ cpm of ^{32}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 $GABA_A$ 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 $GABA_AR$ 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 ^{32}P-labeled primer, separated by gel electrophoresis, and analyzed on a phosphorimager.

RESULTS

To determine whether there are maturational changes in the properties of $GABA_AR$ 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 $GABA_AR$-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 $GABA_ARs$ 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. 1*A*). These were mediated by $GABA_ARs$ based on the demonstration that the mPSPs and mPSCs were reversibly blocked by BMC, a $GABA_AR$ antagonist, but were not affected by the glutamate receptor antagonists CNQX and APV (Fig. 1*A*). In addition, the mPSCs exhibited a reversal potential near the calculated chloride equilibrium potential ($E_{Cl} = -2$ mV) as expected for currents mediated by activation of $GABA_ARs$ (Fig. 1*B*).

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 recorded from $\sim$75% of the neurons examined (Fig. 2*A*). From the second week on, GABAergic mPSCs were present in nearly all of the neurons examined (Fig. 2*A*). 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 $\pm$ SE, $n = 9$) as compared with 2.7 ± 1.01 Hz (mean $\pm$ 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-

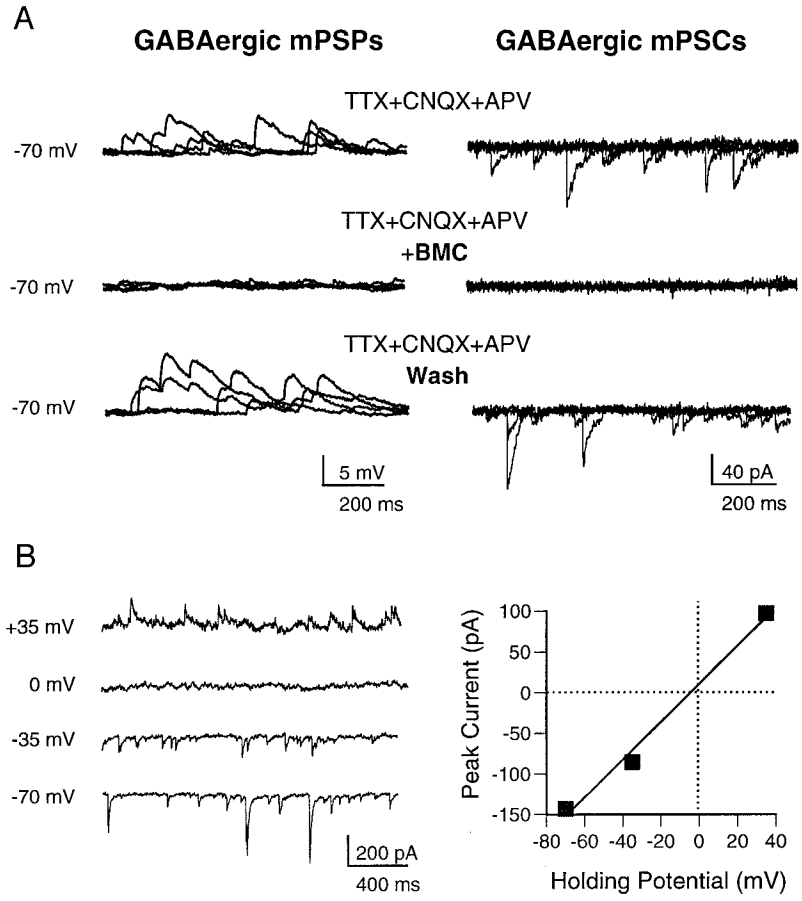

FIG. 1. $GABA_AR$ mediate miniature postsynaptic potentials and currents (mPSPs and mPSCs) recorded from cultured cortical neurons. *A*: superimposed traces (4 1-s sweeps) of mPSPs (*left*) and mPSCs (*right*) obtained from 2 cultured neurons at 21 DIV at a holding potential of −70 mV in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), D(−)-2-amino-5-phosphonopentanoic acid (APV), and tetrodotoxin citrate (TTX). The mPSPs and mPSCs were reversibly blocked by bath application of 2 μM (−)-bicuculline methochloride (BMC). *B*: the mPSCs are inward at negative holding potentials and outward at positive holding potentials. A plot of the average mPSC amplitude vs. holding potential, fit with a linear regression, indicates an *x*-intercept of −4 mV, close to the theoretical equilibrium potential for Cl^-.

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. 3*C*) 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 $GABA_AR$s, 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. 4*A*). 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. 4*A*). 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 ~15 ms during the 4th week (Fig. 4, *B* 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. 4, *D* 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. 4*F*). The mean mPSC decay time constant was significantly faster (3.5-fold) at *P20–P23* when compared with *P8* (Fig. 4*G*). 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. 4*H*). These data suggest that changes in functional properties of synaptically localized $GABA_AR$s contribute to development of inhibition in the rodent neocortex.

Zolpidem sensitivity

The developmental increase in α1 and decrease in α5 $GABA_AR$ subunit expression in the rodent neocortex (Gol-

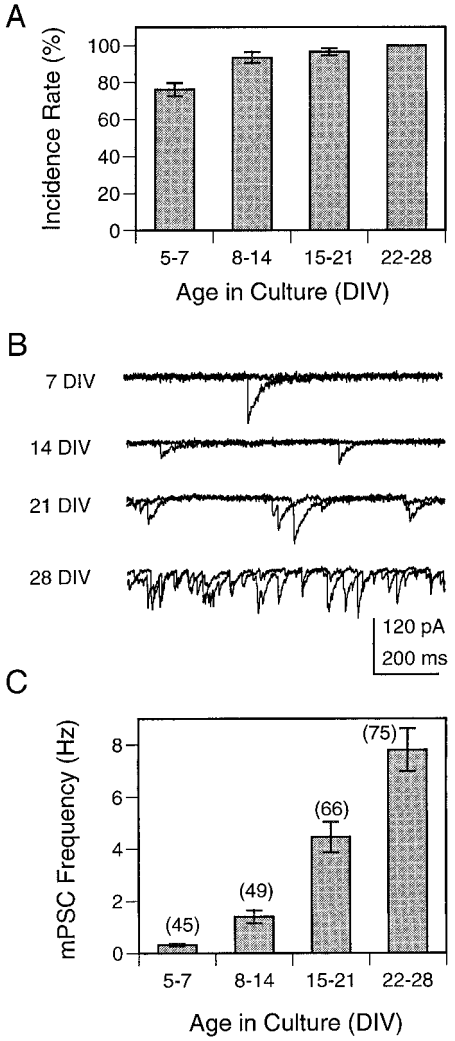

FIG. 2. Developmental increase in incidence and frequency of GABAergic mPSCs. *A*: the incidence rate (percentage of neurons with mPSCs/total number of neurons sampled) is plotted as a function of age. The mean in each age group represents the average incidence calculated from 5 or more experiments in which a minimum of 3 neurons were sampled in each experiment. *B*: representative recordings of mPSCs from 4 different neurons at the indicated ages at a holding potential of −70 mV (2 superimposed 1-s current traces in each sweep). *C*: the mPSC frequency was calculated for each neuron sampled, and the average frequency is plotted as a function of age in culture. Error bars indicate SE, and the number of neurons is in parentheses.

shani et al. 1997; Laurie et al. 1992; Paysan et al. 1994) occurring over the same time period as the changes we report in GABAergic mPSCs, suggests that developmental regulation of these subunits may contribute to the maturational changes in the functional properties of $GABA_A$Rs in cortical neurons. To test this hypothesis we examined the sensitivity of the GABAergic mPSCs to bath application of zolpidem, a benzodiazepine agonist with a high affinity for recombinant $GABA_A$Rs containing α1-subunits compared with receptors containing α5-subunits (Faure-Halley et al. 1993; Macdonald and Olsen 1994; Pritchett and Seeburg 1990). Exposure of a neuron at 28 DIV to 300 nM zolpidem caused a reversible increase in the amplitude of the ensemble average mPSC (Fig. 5*A*). There was also a reversible slowing of the decay time constant, from 8.4 to 13.0 ms, in this same neuron (Fig. 5*C*). Evaluation of mPSCs recorded 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.

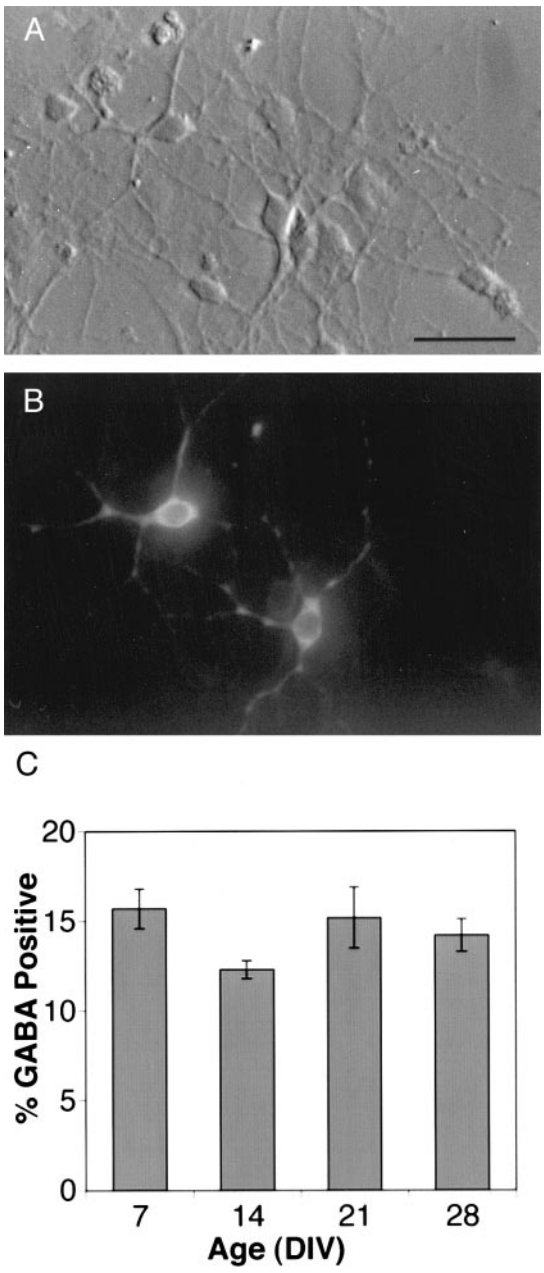

FIG. 3. Subpopulation of GABA-positive neurons in cortical cultures. Cultures were fixed, stained, and visualized by indirect immunofluorescence using an anti-GABA antibody. *A*: photomicrograph of a typical culture fixed at 7 DIV and viewed with Hoffman modulation contrast optics. *B*: epifluorescent 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.

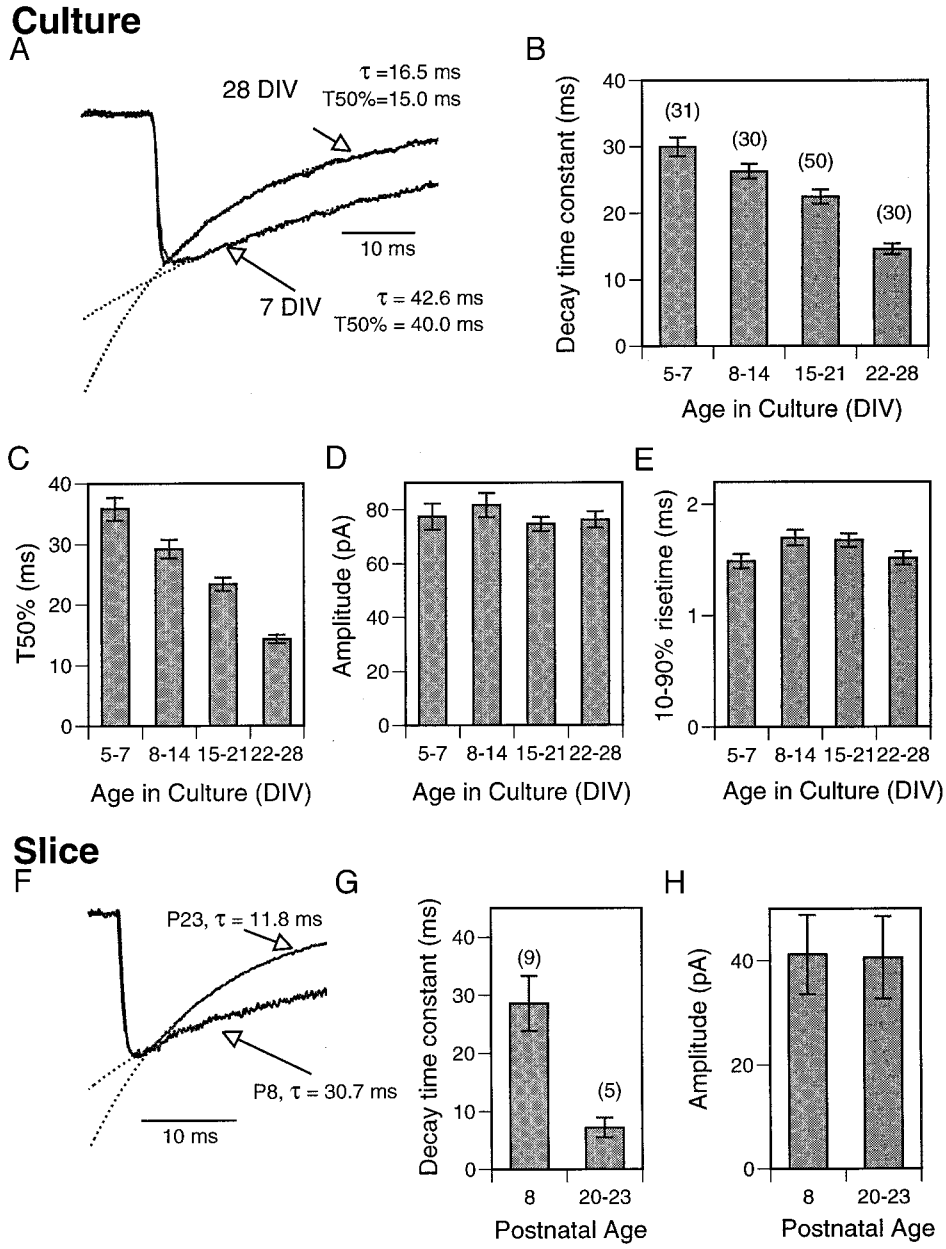

FIG. 4. Increase in rate of decay of GABAergic mPSCs during development in vitro and in vivo. *A*: ensemble average mPSCs (solid lines) recorded from 2 representative neurons at 7 and 28 DIV. Traces were normalized to a unitary amplitude. A single exponential (dashed lines) with the indicated decay time constant (τ) is fit to the data. *B*: the mean mPSC decay time constant decreases significantly as a function of increasing age in culture (ANOVA; $P < 0.0001$). *C*: in the same population of neurons, the mean T50% also decreases significantly as a function of age in culture (ANOVA; $P < 0.0001$). *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 (τ) 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*-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.

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 ($P < 0.05$, unpaired Student's *t*-test; Fig. 5*E*). 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. 5*F*). Taken together, these data suggest that while zolpidem sensitive subunits (e.g., α1) contribute to formation of the $GABA_A$Rs mediating the mPSCs at all ages, their contribution increases during development.

Maturational changes in $GABA_AR$ α-subunit expression

To determine whether an increase in expression of α1 and/or a decrease in expression of α5-subunit mRNA is likely to contribute to developmental regulation of $GABA_A$R function, semiquantitative RT-PCR analysis was used to examine expression of these subunits in neurons developing both in vitro and in vivo. The expression of the individual subunits was assessed in separate RT-PCR reactions using 100 ng total RNA prepared from cultures at 7, 14, 21, and 28 DIV (Fig. 6, *A*, *C*, and *E*). Twenty cycles of amplification using an end-labeled forward primer generated PCR products within the linear range of amplification when quantified on a phosphorimager. RNA harvested from at least three different platings, for each of the developmental ages, was used to quantitatively assess the relative changes in expression of the subunits during the first 4 wk in culture. The most pronounced change was a six- to

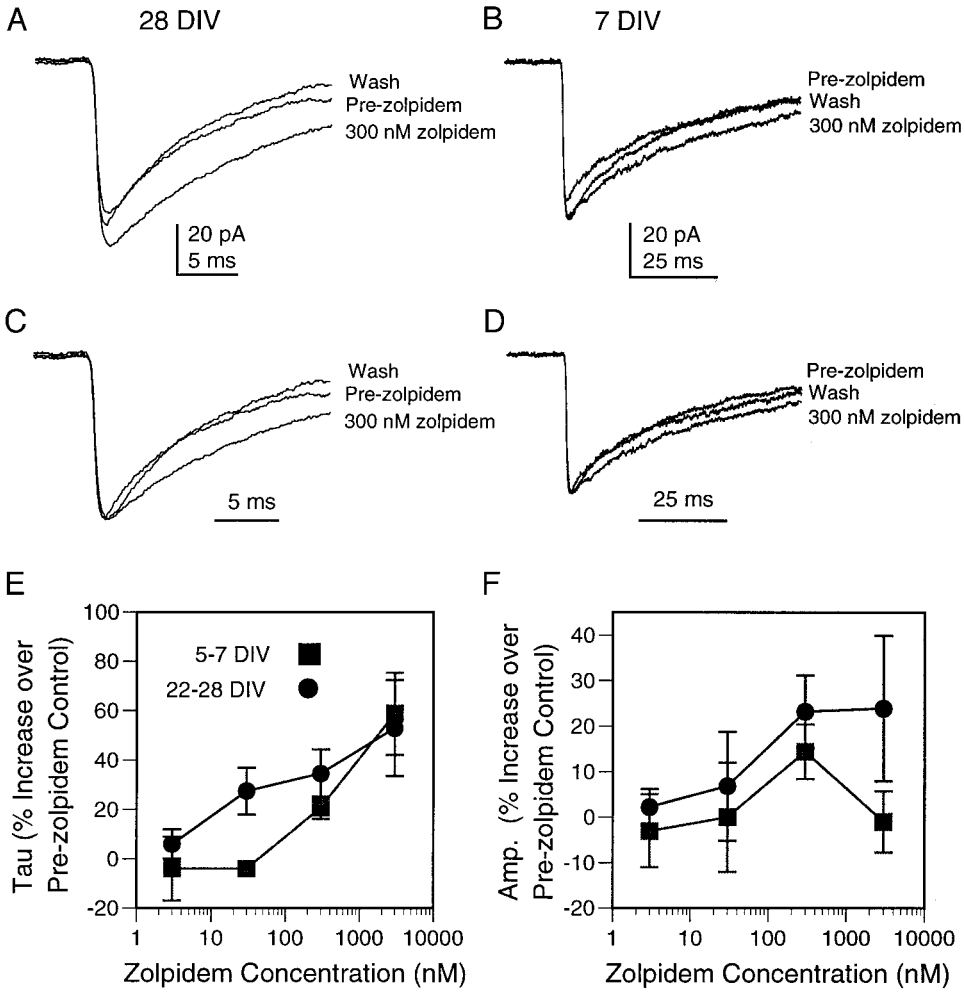

FIG. 5. Zolpidem reversibly increases mPSC amplitude and slows decay in cultured neurons. *A* and *B*: 3 superimposed ensemble average mPSCs recorded from single neurons, 28 and 7 DIV, before (Pre-zolpidem), during (300 nM zolpidem) and after (wash) bath application of zolpidem demonstrate an increase in mPSC amplitude in the presence of zolpidem. *C* and *D*: the reversible slowing of the decay time constant by zolpidem is more clearly visualized in these 2 neurons when the ensemble averages are normalized to the peak current amplitude. *E*: the percent change in decay time constant is plotted as a function of bath concentration of zolpidem. *F*: the percent change in the amplitude is plotted as a function of bath concentration of zolpidem. Each data point represents the mean value determined from 3–17 neurons. Error bars indicate SE.

eightfold increase in the expression of α1 between 7 and 28 DIV ($P < 0.01$, paired Student's *t*-test; Fig. 6*C*). A decrease in α5 expression occurred over this same time period ($P < 0.02$, paired Student's *t*-test; Fig. 6*F*). 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. 7*A*). 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. 7*B*). 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. 8*A*. *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. 8*B*). This was not simply an age-

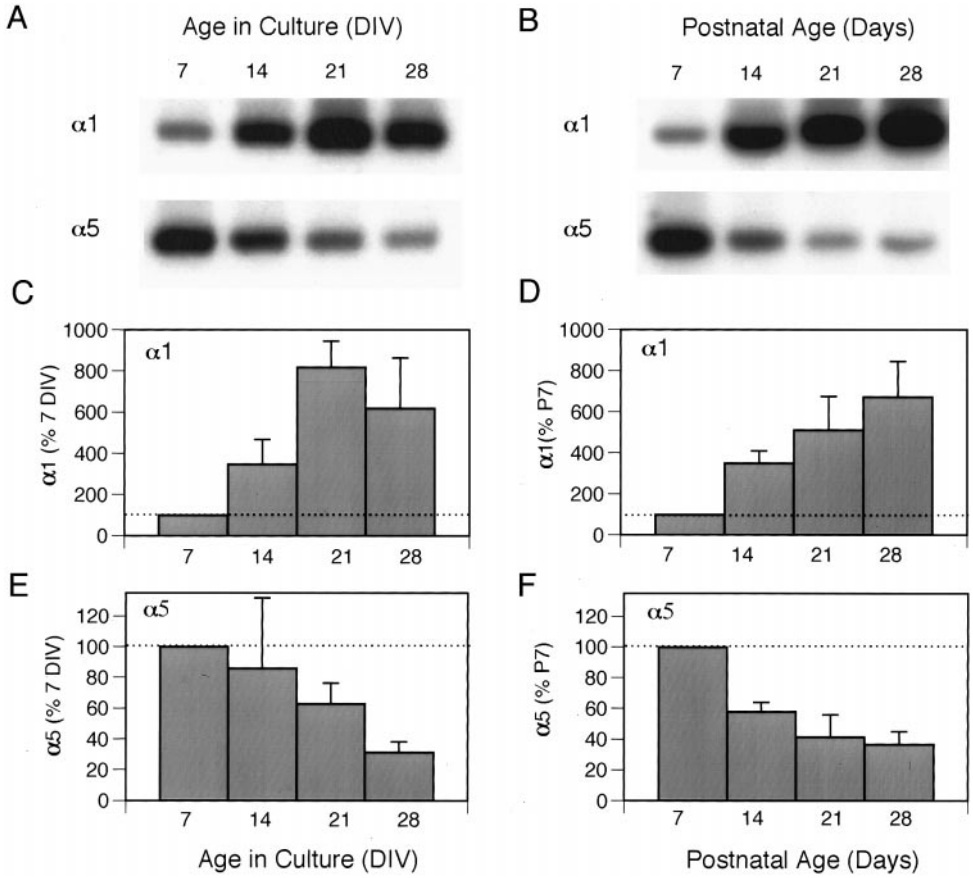

FIG. 6. Developmental changes in $GABA_AR$ α1- and α5-subunit mRNA expression in vitro parallel changes occurring in vivo. *A*: autoradiograms from 2 experiments in which reverse transcription-polymerase chain reaction (RT-PCR) was used to assay the relative levels of expression of α1- or α5-subunits in RNA isolated from cultures at the indicated ages. *C* and *E*: in single RT-PCR experiments a phosphorimager was used to determine the amount of product amplified for each specific α-subunit in RNA harvested from cultures at the indicated ages. The data at 14, 21, and 28 DIV are presented following normalization to the amount of product amplified in a concurrent reaction using RNA harvested at 7 DIV. Mean values represent determinations made from at least 2 independent PCR reactions on 3 separate pools of RNA prepared from cultures at the specified days in vitro, for each subunit. *C*: expression of α1 mRNA increases as a function of age in culture with the peak levels at 21 DIV that are approximately 8-fold higher than those observed at 7 DIV. *E*: α5 expression is similar at 7 and 14 DIV but decreases to ~30% of this value by 28 DIV. *B*: autoradiograms from 2 experiments in which RT-PCR was used to assay the relative levels of expression of α1- or α5-subunits in RNA isolated from somatosensory cortices harvested from animals at the indicated postnatal age. *D* and *F*: developmental changes in subunit expression in vivo quantified using the same method described in *C* and *E*. Mean values represent determination made from independent PCR reactions on 3 separate RNA preparations at each postnatal age. Changes in expression of α1 and α5 in vivo are similar in direction and magnitude during development in vitro. Error bars indicate SE.

dependent phenomenon because a similar correlation was observed when analysis was restricted to neurons in the third week in culture (Fig. 8*C*). In contrast, there was no correlation between α-subunit expression and mPSC amplitude (data not shown). These data support the hypothesis that an increase in the ratio of α1:α5-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 $GABA_AR$s contribute to development of inhibition in the rodent neocortex. Sensitivity of young cultured neurons to low concentrations of zolpidem and the expression of $GABA_AR$ α1-subunits, in RNA harvested from cortical tissue and single neurons in the first week, suggest that α1-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 $GABA_AR$ α1 to α5-subunit mRNA, suggest that an increase in the proportion of synaptic $GABA_AR$s containing α1-subunits contributes to the maturation of GABAergic transmission in mouse cortical neurons.

Maturation in the functional properties of $GABA_AR$s mediating synaptic transmission in cortical neurons

Previous studies have provided evidence of developmental changes in the functional properties of synaptically localized $GABA_AR$s 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

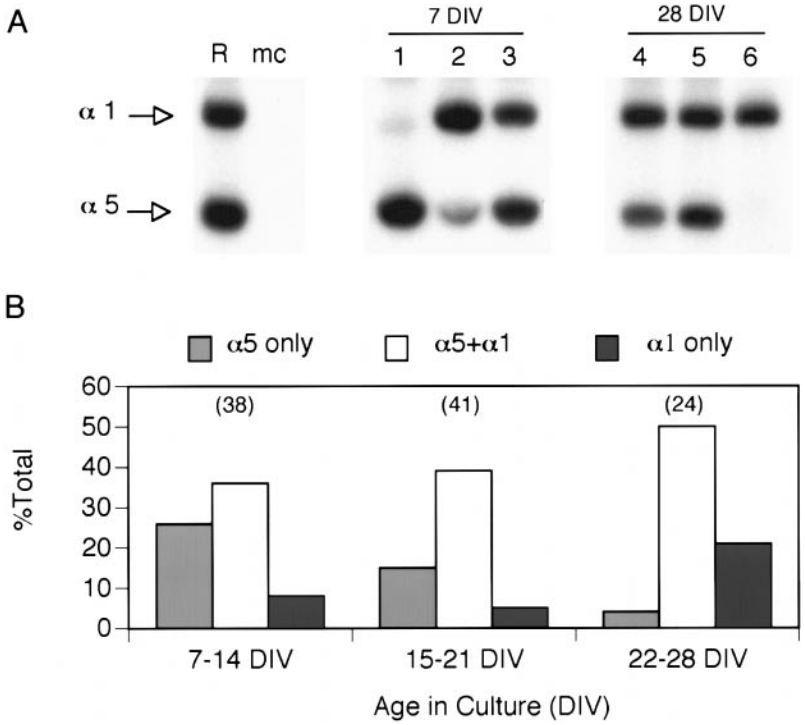

FIG. 7. Developmental change in the frequency of single neurons in which only α1- or only α5-subunit mRNA was detected. Products unique for α1- and/or α5-subunit mRNA were amplified following reverse transcription and 2 rounds of multiplex PCR amplification using 2 nested primer sets specific for the 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 α5 was amplified from *cell 1* at 7 DIV, and only α1 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 α1 and α5 mRNA. However, the frequency of cells in which only α5 was amplified was highest between 7 and 14 DIV, whereas the frequency of cells in which only α1 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.

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 $GABA_AR$ 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 mPSCs in cortical neurons developing in dissociated cell culture are similar to

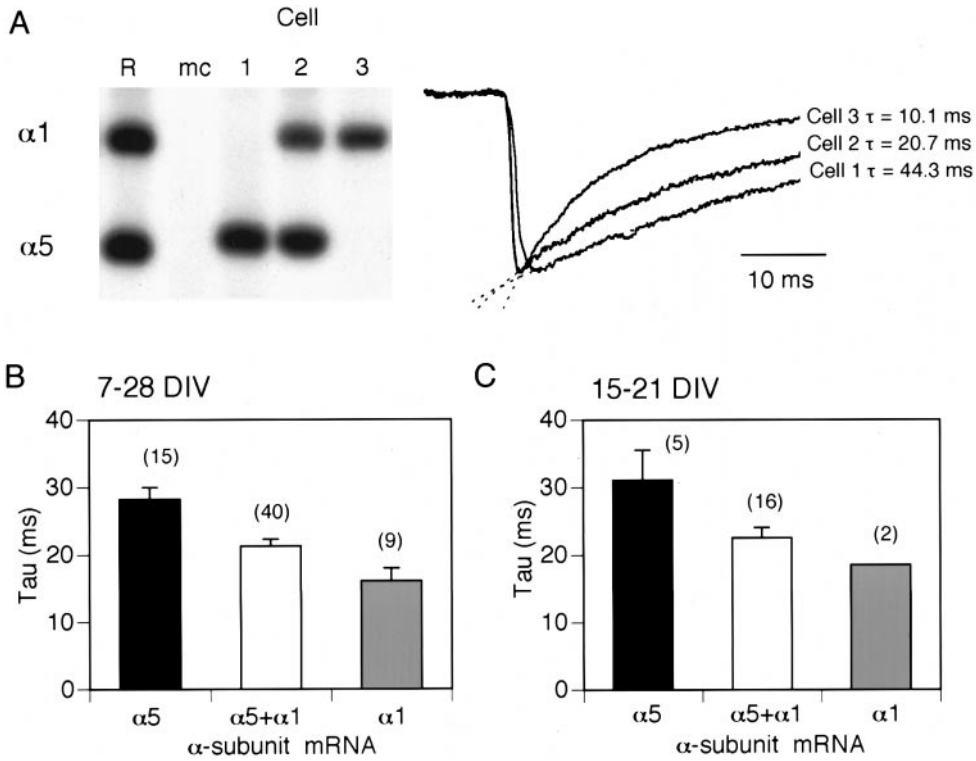

FIG. 8. Correlation between expression of α-subunit expression and mPSC decay time constant in single neurons. *A*: autoradiogram of the PCR products amplified from RNA (R) harvested from a whole culture and from 3 individual neurons in RT-multiplex PCR reactions. Media control (mc). The averaged mPSCs (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 α5 was detected, had the slowest decay time constant, whereas *cell 3*, in which only α1 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 α5 only was detected was significantly longer than that seen in $\alpha1 + \alpha5$ (ANOVA; $P < 0.01$ Fishers PLSD) and in α1 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.

that seen in vivo demonstrates that alterations in sensory input are not necessary to initiate the change.

Although GABAergic mPSCs get faster during the first 4 wk of development, the magnitude of the change we observed was greater in cortical neurons maturing in vivo versus in vitro. A number of variables may contribute to this difference. It is possible, for example, that although the signals necessary for initiating the changes in $GABA_AR$ function are intrinsic to the cortex, or even cell autonomous, additional factors important in completion and/or maintenance of the more differentiated state may be absent in the cultures. Recent studies have also demonstrated differences in the decay kinetics of GABAergic sIPSCs between pyramidal and interneurons in the rodent cortex, providing evidence of cell-specific expression of functionally distinct $GABA_ARs$ (Xiang et al. 1998). In the present study, GABAergic mPSCs were recorded from randomly selected neurons in the culture dish and within layers V and VI in the slice preparations. Thus sampling from different subpopulations could also contribute to the differences in mean mPSC decay time between neurons examined in culture and slice.

α1-Subunits contribute to functional GABAergic mPSCs during the first postnatal week

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; Soltesz 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 $GABA_AR$-mediated synaptic currents in the first week. In addition, our single-cell RT-mPCR analysis revealed that both α1- and α5-subunits could be amplified from the majority of neurons examined, even in 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 $GABA_AR$ α-subunit expression contribute to the development of GABAergic transmission in cortical neurons

An increase in the expression of the α6 $GABA_AR$ 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 $GABA_AR$-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 Soltesz 1997; Kapur and Macdonald 1999; Rovira and Ben-Ari 1993). In this study, we present three lines of 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 vitro is consistent with an increase in the contribution of α1-subunits to $GABA_ARs$ 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 mIPSC 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 $GABA_ARs$.

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) $GABA_AR$ 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 (Paysan et al. 1997) and receptor activation (Poulter et al. 1997) can regulate $GABA_AR$ subunit expression in developing cortical neurons. Thus continued exploration of the functional and molecular properties of $GABA_ARs$ 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.

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Address for reprint requests: D. K. O'Dowd, Dept. of Anatomy and Neurobiology, University of California, Irvine, CA 92697-1280.

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