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GABAergic Signaling to Newborn Neurons in Dentate Gyrus

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Overstreet Wadiche, Linda, Daniel A. Bromberg, AeSoon L. Bensen, and Gary L. Westbrook. GABAergic signaling to newborn neurons in dentate gyrus. *J Neurophysiol* 94: 4528–4532, 2005. First published July 20, 2005; doi:10.1152/jn.00633.2005. Neurogenesis in the dentate gyrus begins before birth but then continues into adulthood. Consequently, many newborn granule cells must integrate into a preexisting hippocampal network. Little is known about the timing of this process or the characteristics of the first established synapses. We used mice that transiently express enhanced green fluorescent protein in newborn granule cells to examine their synaptic input. Although newborn granule cells had functional glutamate receptors, evoked and spontaneous synaptic currents were exclusively GABAergic with immature characteristics including slow rise and decay phases and depolarized reversal potentials. Synaptic currents in newborn granule cells were relatively insensitive to the $GABA_A$ receptor modulator zolpidem compared with neighboring mature granule cells. Consistent with the kinetics and pharmacology, newborn granule cells isolated by fluorescent cell sorting lacked the $\alpha 1$ $GABA_A$ receptor subunit. Our results indicate that newborn granule cells initially receive only GABAergic synapses even in the adult.

INTRODUCTION

Newborn neurons are continuously generated from neural progenitors in the dentate gyrus of adult mammals. Over the course of several months adult-generated cells can develop into mature neurons (van Praag et al. 2002). Yet little is known about the progression of neuronal maturation in the environment of the adult brain. In the neonatal hippocampus, developing pyramidal cells initially receive GABAergic input followed sequentially by *N*-methyl-D-aspartate (NMDA) and AMPA receptor-mediated synaptic responses (Tyzio et al. 1999). The slow kinetics and depolarizing reversal potentials of immature GABAergic synapses could facilitate trophic actions of GABA during development (Owens and Kriegstein 2002). However, it is unclear whether adult-generated granule cells follow a similar developmental progression. Here we used a transgenic mouse that transiently expresses enhanced green fluorescent protein in newborn granule cells (Overstreet et al. 2004b) to show that adult-generated granule cells receive GABAergic synapses with properties characteristic of immature synapses. Our results indicate that adult-generated granule cells initially receive GABAergic input from local interneurons, and remain isolated from extrinsic excitatory input during the first 2 wk of their existence.

METHODS

Animals

Transgenic −13/+8POMC-EGFP mice were obtained as described (Overstreet et al. 2004b). We used heterozygous −13/+8POMC-EGFP mice maintained by out-breeding homozygous males with wild-type C57BL/6J females. All animal procedures followed the Guide for the Care and Use of Laboratory Animals, United States Public Health Service and were approved by the OHSU IACUC.

Electrophysiology

POMC-EGFP mice from 4 days postnatal to 6 mo of age were used for experiments. Horizontal slices from the hippocampus were incubated in a solution containing (in mM) 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 2.5 $MgCl_2$, and 25 D-glucose, bubbled with 95% O_2-5% CO_2. For whole cell recordings, $CaCl_2$ was increased to 2.0 mM and $MgCl_2$ was reduced to 1.0 mM. Patch pipettes were filled with (in mM) 150 KCl, 10 HEPES, 4 Mg_2ATP, 0.5 NaGTP, 10 phosphocreatine, and 0.2% biocytin (pH 7.3 and 310 mosM, 4–8 MΩ resistance). Exogenous agonists were applied via a puffer pipette placed near the cell soma (20 ms, 3–6 psi, Picospritzer, General Valve, Fairfield, NJ). In these experiments, the intracellular solution contained 10 mM EGTA. Differential interference contrast and fluorescent images were combined (PIX/2, MicroImage Video Systems, Boyertown, PA) for simultaneous viewing of $EGFP^+$ and unlabeled cells. Series resistance (8–25 MΩ) was monitored, and experiments were discarded if substantial changes were observed. Currents were filtered at 2 kHz and sampled at 10 kHz (Axopatch 200B, Molecular Devices, Union City, CA). We evoked synaptic responses with a saline-filled pipette placed in the medial perforant path or granule cell layer. Spontaneous activity was recorded in 15-min epochs and analyzed off-line using Axograph 4.9. Unless noted, recordings were done at 22°C. Tonic $GABA_A$ receptor-mediated activity was measured by the reduction in baseline current noise caused by SR95531 (Overstreet and Westbrook 2001). For perforated-patch recordings, the intracellular solution contained 150 KCl, 10 HEPES, and 2 MgCl with 50 μg/ml gramicidin, and extracellular solutions contained 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[f]quinoxaline-7-sulfonamide, 2-amino-5-phosphonopentanoic acid (AP5), and (25)-3[[(15)-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl) phoshinic acid. Voltages were corrected by $V_{corrected} = V_{command} - I * R_s$, where I is the peak current from baseline and R_s is the series resistance (20–80 MΩ). Voltages were not corrected for Donnan potentials. Data are expressed as means ± SE. Unless noted, paired or unpaired *t*-test were used to determine statistical significance at the $P < 0.05$ level. All drugs and chemicals were obtained from Sigma or Tocris.

Fluorescent-activated cell sorting

POMC-EGFP mice were anesthetized with halothane. The dentate gyrus was micro-dissected away from the hippocampus and digested with papain (20 U/ml) at 37°C for 45 min. The reaction was terminated with bovine serum albumin/trypsin inhibitor/heat inactivated fetal calf serum (HIFCS). The cell suspension was triturated in 1%HIFCS, passed through a 35-μm nylon mesh filter, and resuspended at 1×10^6 cells/ml. DNase I (10 U/ml) was added to reduce

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clumping. Cells were sorted on a flow cytometer (FACS Vantage Diva, BD Biosciences, San Jose, CA) equipped with an argon-ion laser (488 nm and 200 mW) operated at 10-psi and 20,000 drops/s with a 130-μm tip. EGFP fluorescence was detected using a 530/30-nm band-pass filter. Cells were sorted into culture tubes containing 1 ml tissue culture media at a rate of 2,000–4,000 cells/s. Postsort histograms revealed that the sorted cells were 99% pure. cDNA was synthesized from sorted cells with SuperScript III kit (Invitrogen). PCR was performed on the synthesized cDNA from sorted $EGFP^+$ and $EGFP^-$ cells. Reactions were started at 94°C for 1 min then cycled 40 times at 94°C for 30 s, 55°C for 30 s, and 71°C for 1 min. Each reaction contained 1 μl of the forward and reverse primer, 2 μl of cDNA in Platinum Supermix (Invitrogen) containing 2 U Platinum taq polymerase (Invitrogen). Published primer sequences against $GABA_A$ receptor α subunits were used (Alsbo et al. 2001; Gustincich et al. 1999). PCR products were run on a 2% agarose gel containing 761 nM ethidium bromide.

RESULTS

Early synapses on newborn granule cells are exclusively GABAergic

EGFP expression in the dentate gyrus of POMC-EGFP mice labels newborn granule cells with immature morphology and excitable properties in both adults and neonates (Overstreet et al. 2004a,b). Puff application of AMPA and NMDA revealed that EGFP-labeled newborn granule cells expressed functional glutamate receptors (not shown), but responses to glutamate receptor agonists were much smaller than agonist-evoked GABA responses (Fig. 1*A*). Furthermore, stimulation of the medial perforant path in the presence of $GABA_A$ receptor antagonists did not produce synaptic responses ($n = 24$), whereas robust excitatory postsynaptic currents (EPSCs) were evoked in neighboring mature granule cells (not shown). Stimulation of the inner molecular layer and granule cell layer also failed to evoke glutamatergic synaptic currents ($n = 15$). Thus newborn granule cells received little or no excitatory synaptic input from extrahippocampal sources. The lack of extrinsic input is consistent with their short dendrites that do not extend into the outer 2/3 of the molecular layer where the perforant path terminates (Overstreet et al. 2004b).

However, newborn neurons were surrounded by a dense network of GABAergic synaptic boutons (Fig. 1*B*), suggesting that they might receive GABAergic synapses. Indeed, stimulation in the medial perforant path evoked small synaptic currents (36 ± 7 pA, $n = 6$) with slow rise times (8.0 ± 0.8 ms) and durations (half-width: 87 ± 6 ms) that were blocked by SR95531 ($n = 6$, Fig. 1*C*). These GABAergic IPSCs were also reduced by CNQX (to 38 ± 19% of control, $n = 3$), indicating they were partially mediated by polysynaptic excitation of local GABAergic interneurons (Liu et al. 1998). Stimulation within the granule cell layer evoked monosynaptic IPSCs (121 ± 33 pA, $n = 4$) that reversed near the calculated Cl^- reversal potential (3.2 mV, Fig. 1*D*) and had slow rise

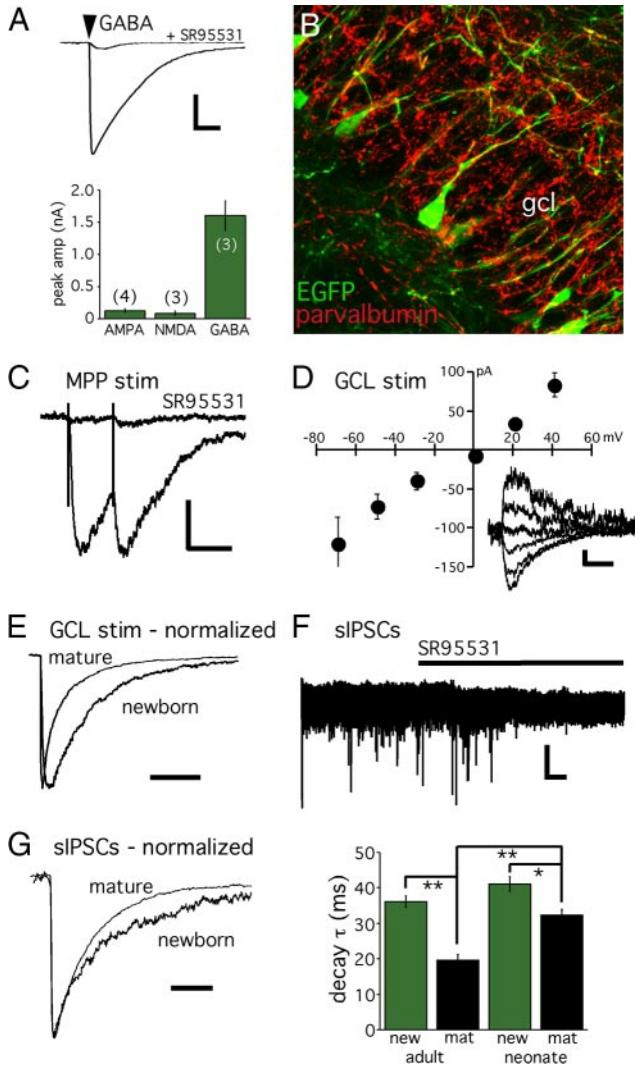

FIG. 1. Early synapses on newborn granule cells are exclusively GABAergic. *A*: puff application of GABA (1 mM) evoked robust responses in newborn granule cells. Scale bar, 500 pA, 1 s. GABA-evoked currents were blocked by SR95531 (10 μM, $n = 3$). GABA evoked 10-fold larger peak currents than AMPA (200 μM) or *N*-methyl-D-aspartate (NMDA, 100 μM). Recordings were made in slices from postnatal day (PD) 14–22 mice. AMPA- and NMDA-evoked responses in newborn granule cells had similar amplitudes and current-voltage relationships to responses in neighboring mature granule cells (not shown). The numbers in parenthesis indicate the number of cells. *B*: expression of a marker for basket cells (parvalbumin, red) shows that newborn neurons (green) in the granule cell layer (gcl) were surrounded by a dense network of GABAergic synaptic boutons. *C*: synaptic currents in newborn granule cells evoked by stimulation of the medial perforant path (MPP) were completely blocked by the $GABA_A$ receptor antagonist SR95531 (5 μM). Scale bars 20 pA, 100 ms. Recordings were made in slices from adult mice (>3 mo old). *D*: synaptic currents in newborn granule cells evoked by stimulation of the granule cell layer (GCL) evoked monosynaptic inhibitory postsynaptic currents (IPSCs) that reversed near the calculated Cl^- reversal potential of 0 mV [KCl intracellular, in 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX) and 2-amino-5-phosphonopentanoic acid (AP5), 32°C, $n = 4$]. *Inset*: IPSCs recorded at the indicated membrane potentials. Scale bars, 20 pA, 50 ms. *E*: monosynaptic IPSCs in newborn granule cells had small amplitudes, as well as slower rise times and decay phases compared with IPSCs evoked in neighboring mature granule cells. Averaged IPSCs were normalized to peak currents. Scale bar, 50 ms. Recordings were made in slices from postnatal day 14–22 mice at 32°C. *F*: spontaneous events in newborn granule cells were blocked by SR95531 (5 μM; no other antagonists present). The amplitude of spontaneous IPSCs (sIPSCs) in newborn cells (34 ± 13 pA, $n = 7$) was smaller than in neighboring mature granule cells (67 ± 9 pA, $n = 7$). Scale bar, 10 pA, 20s. *G*: spontaneous events recorded in CNQX and AP5 were averaged and normalized. sIPSCs in newborn neurons decayed more slowly than sIPSCs in neighboring mature granule cells. Recordings were done at 22°C. Scale bar, 20 ms. Decays were fit with two exponentials and described by the weighted average. The decay of sIPSCs in newborn neurons were the same regardless of the age of the animal, but were slower than sIPSCs in unlabeled mature neurons in adults and neonates (ANOVA with Bonferroni multiple comparisons, * $P < 0.01$; ** $P < 0.001$).

times (6.1 ± 1.5 ms) and prolonged half-widths (41 ± 5 ms) compared with IPSCs in neighboring mature granule cells (780 ± 205 pA, 0.83 ± 0.12 ms, 15 ± 1.6 ms, respectively, $n = 7$, Fig. 1*E*). Newborn granule cells in adults and neonates had a low frequency of spontaneous IPSCs (sIPSCs) that were completely blocked by SR95531 (Fig. 1*F*); a fraction of cells lacked detectable events (27 and 28%, respectively). The sIPSC frequency in newborn cells in adults (0.04 ± 0.02 Hz, $n = 7$) and neonates (0.06 ± 0.03 Hz, $n = 11$) was much lower than in mature granule cells in adults (7.0 ± 0.7 Hz, $n = 5$) and neonates (6.5 ± 1.9 Hz, $n = 5$). The weighted decay time constant for sIPSCs in newborn cells was 36 ± 1.6 ms in adults ($n = 5$) and 41 ± 2 ms in neonates ($n = 5$; Fig. 1*G*). These decays were significantly slower than in neighboring mature granule cells in adults (20 ± 1.6 ms, $n = 5$) and neonates (32 ± 1.6 ms, $n = 8$). Together these results indicate newborn granule cells receive GABAergic synaptic input with slow kinetics in both adults and neonates, albeit at a low frequency compared with neighboring mature granule cells. In addition, SR95531 (5 μM) reduced the baseline current noise (to 70 ± 8% of control, $n = 13$) in newborn granule cells from adults and neonates, suggesting ambient levels of GABA can persistently activate $GABA_A$ receptors (Farrant and Nusser 2005).

Depolarized GABA responses in newborn granule cells

Elevated intracellular [Cl^-] in immature neurons can result in depolarizing GABAergic responses and may underlie a trophic role for GABA during development (Owens and Kriegstein 2002). At postnatal day 16–22 when the reversal potential for GABA-activated currents (E_{Cl}) was –74 ± 7 mV in mature granule cells ($n = 6$), perforated-patch recordings revealed that E_{Cl} in newborn neurons was much more depolarized (−45 ± 2 mV, $n = 7$, $P < 0.001$, Fig. 2*A*). Rupture of membrane patches shifted the reversal potential to 1.0 ± 0.5 mV ($n = 11$), near the calculated E_{Cl} for the intracellular solution.

Although GABAergic responses in newborn neurons were relatively depolarized, GABA application evoked action potentials only in one of six cells (not shown). In most cells, the threshold for action potential generation, measured by current injections (−38 ± 2 mV, 5 of 6 cells), was more positive than E_{Cl} (−47 ± 4 mV, $n = 5$). GABA application shunted the membrane conductance, clamping it at E_{Cl} and thus blocked action potentials elicited by current injection (Fig. 2*B*). Newborn cells had depolarized resting membrane potentials (−43 ± 2 mV, $n = 7$) and sometimes fired spontaneously. In these cases, GABA inhibited spontaneous firing (Fig. 2*C*). Thus GABAergic responses in newborn neurons are depolarizing but do not necessarily generate sodium-dependent action potentials.

Newborn granule cells lack the $GABA_A$ receptor α1 subunit

The slow decay of IPSCs in adult-generated granule cells could reflect altered subunit composition of synaptic receptors. During neonatal development, incorporation of the α1 subunit speeds the decay of IPSCs and renders them more sensitive to the imidazopyridine zolpidem (Dunning et al. 1999; Hollrigel and Soltesz 1997; Ortinski et al. 2004; Vicini et al. 2001). Consistent with this pattern, sIPSCs in newborn granule cells were less sensitive to zolpidem (0.5 μM) than neighboring

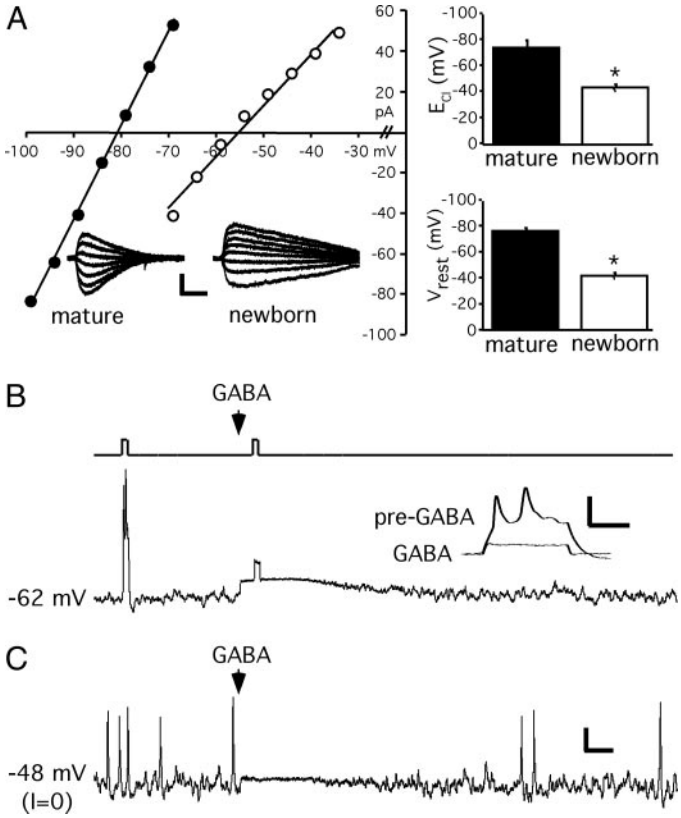

FIG. 2. GABA responses are depolarizing, but shunt action potentials. *A*: current-voltage relationship of GABA-evoked currents in mature and neighboring newborn granule cells in perforated-patch recordings from a PD 22 mouse (*left*). *Insets*: currents evoked by GABA (200 μM) at the displayed voltages. Scale bars, 40 pA, 400 ms. The reversal potential for GABA-evoked currents (E_{Cl}) and the resting membrane potential (V_{rest}) were depolarized in newborn granule cells compared with neighboring mature cells (*right*). It is likely that the measured resting potential of newborn cells was artificially depolarized due to the high-input resistance (9.5 ± 1.4 GΩ, $n = 7$) (Pongracz et al. 1991). *B*: in a newborn granule cell, current injections (*top,* 200 ms, 5 pA) evoked TTX-dependent action potentials that were shunted by GABA (aligned in *inset*, scale bars, 20 mV, 100 ms). The cell was held near −60 mV in current clamp. In 5/6 cells, E_{Cl} was more negative than the firing threshold so that GABA-evoked depolarization did not elicit action potentials. *C*: at its resting potential (−48 mV), a newborn cell fired spontaneous action potentials that were eliminated by a GABA puff. Scale bar, 10 mV, 1 s.

mature cells at PD 21–25 (Fig. 3*A*). The weighted decay of sIPSCs was 6.6 ± 0.7 ms in mature cells ($n = 3$, 34°C) compared with 16.9 ± 3.6 ms in newborn cells ($n = 3$). Zolpidem prolonged the decay of sIPSCs in mature cells by 67 ± 13% but only by 24 ± 6% in newborn cells ($n = 3$, $P < 0.03$).

To determine whether the $GABA_A$ receptor subunit composition in newborn granule cells differed from mature granule cells, we used fluorescence-activated cell sorting (FACS) to isolate $EGFP^+$ and $EGFP^-$ granule cells. The dentate gyrus was microdissected from the hippocampus so that $EGFP^-$ cells largely consisted of mature granule cells. Primers against α subunits (Alsbo et al. 2001; Gustincich et al. 1999) were first confirmed in whole-brain extracts (not shown). As expected (Wisden et al. 1992), all subunits except for the α6 were detected in the population of mature granule cells, even as early as PD 6 (Fig. 3*B*). However, newborn cells had a different expression profile. Consistent with the reduced zolpidem sensitivity, newborn cells lacked α1 subunits and α3 but expressed α2 and α5 (Fig. 3*C*). At PD 20–21, an age when the

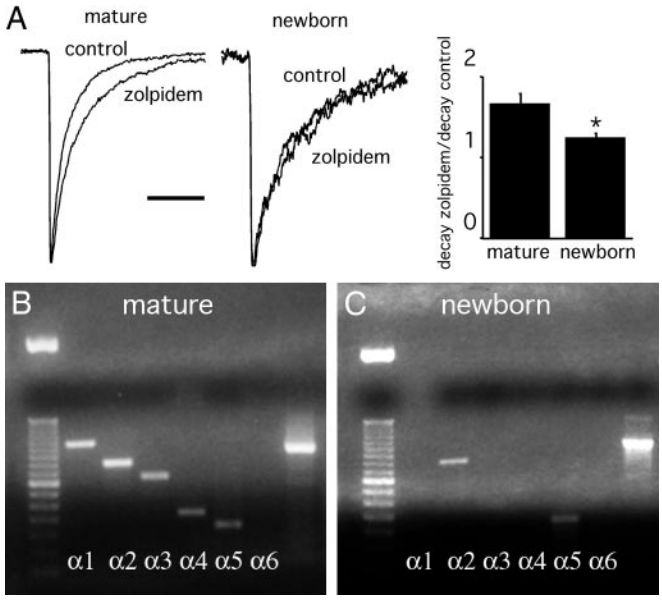

FIG. 3. Newborn granule cells lack the $GABA_A$ receptor α1 subunit. *A*: zolpidem prolonged sIPSCs in mature granule cells to a greater degree than newborn granule cells. Averaged and normalized sIPSCs in a mature and a newborn cell in control and zolpidem (0.5 μM). Scale bar, 20 ms. The amplitude of sIPSCs in newborn granule cells (28 $\pm$ 5 pA) was smaller than sIPSCs in neighboring mature granule cells (69 $\pm$ 6 pA, n = 3 each). Recordings were done in slices from P21 to P24 mice at 32°C. *B*: $EGFP^-$ mature granule cells isolated by FACS expressed α1–α5 $GABA_A$ receptor subunits (*left*). Consistent with their reduced zolpidem sensitivity, α1 subunits were not detected in isolated newborn granule cells (*C*). Only α2, α4 (not shown), and α5 (barely detectable) subunits were detected in newborn cells. Each gel contains a 50-bp ladder in the first lane and a β-actin control reaction in the last lane.

decay kinetics of IPSCs in mature granule cells has reached adult values (Hollrigel and Soltesz 1997), similar results were obtained except the α4 subunit was also detected in some batches of newborn cells (not shown). The lack of α1 expression likely underlies the slow decay of GABAergic synaptic currents and the reduced zolpidem sensitivity in newborn granule cells.

DISCUSSION

EGFP expression in POMC-EGFP mice labels adult-generated granule cells ~2 wk after mitosis (Overstreet et al. 2004b). Newborn granule cells appear to be at a similar functional stage in adults and neonates. However, granule cell development is accelerated in neonates such that labeled cells are ~1 wk postmitotic (Overstreet et al. 2004a). Hence, newborn neurons in neonates receive exclusively GABAergic synapses for at least a week after cell division, whereas in adults, they are exclusively present for ~2 wk. Although there was no detectable glutamatergic input, we cannot completely exclude that newborn cells could have "silent" synapses resulting from an absence of postsynaptic AMPA receptors (Wu et al. 1996) or tonic presynaptic inhibition (Moore et al. 2003). Regardless, at the time of EGFP expression, newborn granule cells receive input from local inhibitory interneurons, but they are isolated from direct extrinsic input from the entorhinal cortex.

Our results are consistent with GABAergic synaptic input to adult-generated granule cells that have been identified on the basis of their physiological properties and morphology (Ambrogini et al. 2004). Recent work has also revealed GABAergic synaptic currents in a small population of nestin-GFP-labeled cells (Wang et al. 2005). This subpopulation of "type 2" cells lacked an apical dendrite and axon, suggesting that they are more immature than the EGFP-labeled cells studied here. Nestin-GFP cells with neuronal properties could represent the earliest stage of neuronal differentiation (Kempermann et al. 2004), at a time when nestin expression has ceased but GFP fluorescence is still detectable. The larger proportion of POMC-EGFP newborn cells with spontaneous synaptic currents (~75%) compared with nestin-EGFP type 2 cells (~13%) suggests that the acquisition of GABAergic synapses is an important step in the maturation of adult-generated neurons.

The expression of $GABA_A$ receptor subunits throughout the brain changes during development (Laurie et al. 1992). In dentate granule cells, expression of α1, α3, and α4 increases, whereas α5 subunit expression decreases (Brooks-Kayal et al. 2001). α1-containing receptors have fast deactivation kinetics (Gingrich et al. 1995; Verdoorn 1994), and their inclusion at synapses underlies the acceleration of IPSCs during development (Ortinski et al. 2004; Vicini et al. 2001). Likewise we found that newborn granule cells had slow IPSCs and lacked α1 subunits. Although previous work suggests the age of the animal rather than the age of the neuron is critical for determining the decay of IPSCs (Hollrigel and Soltesz 1997), our results indicate newborn cells have these immature characteristics at all ages of the animal. For example, at PD 21–24, newborn cells were less sensitive to the α1-selective agent zolpidem compared with neighboring mature cells.

Mounting evidence indicates that early in development GABA can have trophic effects on cell proliferation, migration, and neurite outgrowth (Ben-Ari 2002; Owens and Kriegstein 2002). A depolarized reversal potential of GABA-induced responses is thought to underlie its trophic actions, with GABA itself regulating the developmental switch from depolarization to hyperpolarization (Ganguly et al. 2001; but see Ludwig et al. 2003; Titz et al. 2003). GABA could exert trophic effects either via conventional synaptic responses or by tonic activation of extrasynaptic receptors (Demarque et al. 2002; Manent et al. 2005). Tonic GABAergic signaling in dentate granule cells is mediated primarily by extrasynaptic receptors containing δ subunits (Stell et al. 2003) that may assemble with α4 subunits (Sun et al. 2004; Sur et al. 1999). We detected α4 subunits in one batch of $EGFP^+$ cells, and many newborn granule cells had tonic $GABA_A$ receptor-mediated activity. The very high-input resistance of newborn granule cells (Overstreet et al. 2004b) would allow a low level of tonic activation to influence their excitability. The slow kinetics of IPSCs in newborn neurons could enhance trophic actions of GABA by facilitating GABA-induced depolarization and thus intracellular $[Ca^{2+}]$ transients. Although GABA responses did not generate Na^+-dependent action potentials in newborn neurons, the depolarization was past the threshold for activating Ca^{2+} channels. Spontaneous Ca^{2+} transients do occur in newborn cells in neonates (Overstreet Wadiche and Westbrook, unpublished observations).

The appearance of GABAergic synaptic events in adult-generated granule cells before glutamatergic activity arises is similar to the sequence of events in CA1 pyramidal cells during neonatal development (Tyzio et al. 1999). Yet this sequence contrasts with synaptogenesis in the progeny of adult stem cells

in vitro, where glutamatergic synapses dominate (Song et al. 2002). This discrepancy highlights the importance of in vivo studies, where structural constraints determine how developing dendritic arbors interface with available nerve terminals. Modulation of the depolarizing GABAergic responses in newborn granule cells could profoundly influence their maturation and possibly survival.

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