GABA Actions in Hippocampal Area CA3 During Postnatal Development: Differential Shift From Depolarizing to Hyperpolarizing in Somatic and Dendritic Compartments

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Romo-Parra H, Treviño M, Heinemann U, Gutiérrez R. GABA actions in hippocampal area CA3 during postnatal development: differential shift from depolarizing to hyperpolarizing in somatic and dendritic compartments. J Neurophysiol 99: 1523–1534, 2008. First published January 23, 2008; doi:10.1152/jn.01074.2007. γ-Aminobutyric acid type A receptor (GABA_A-R) activation leads to depolarization of pyramidal cells during the first postnatal week and produces hyperpolarization from the second week. However, immunohistochemical evidence has suggested that during the second and third postnatal weeks the NKCC1 cotransporter relocates from the soma to the dendrites of CA3 pyramidal cells. We hypothesized that this leads to depolarizing responses in apical dendrites. Here we show that the activation of GABA_A-R in the distal dendrites of CA3 pyramidal cells at P15 by restricted application of muscimol or synaptic activation by stimulation of interneurons in stratum radiatum (SR) causes depolarizing postsynaptic potentials (PSPs), which are blocked by NKCC1 cotransporter antagonists. By contrast, activation of proximal GABA_A-R by muscimol application or by stimulation of interneurons in stratum oriens (SO) leads to hyperpolarizing PSPs. Activation of the dentate gyrus (DG) in the presence of glutamatergic blockers evokes hyperpolarizing responses during the second postnatal week; however, the reversal potential of the DG-evoked inhibitory (IPSPs) is more depolarized than that of IPSPs evoked by activation of SO interneurons. Despite the shift of GABA action from depolarizing to hyperpolarizing, DG-evoked field potentials (f-PSPs) are not changed in polarity until the third week. Current source density analysis yielded results consistent with depolarizing actions of GABA in the dendritic compartment. Our data suggest that GABAergic input to apical dendrites of pyramidal cells of CA3 evokes depolarizing PSPs long after synaptic inhibition has become hyperpolarizing in the somata, in the axon initial segments and in basal dendrites.

INTRODUCTION

The inhibitory effect of γ-aminobutyric acid (GABA) acting on GABA_A receptors (GABA_A-Rs) in the adult brain is due to membrane hyperpolarization following the influx of Cl− through the ionophore and the shunting of dendritic and somatic excitatory inputs. However, in the immature brain E_GABA is positive with respect to the resting membrane potential and, consequently, the activation of the GABA_A-R depolarizes the neuronal membrane through the outward flow of Cl− (Cherubini et al. 1991; Owens et al. 1996; Rivera et al. 1999). Indeed, during this period the NKCC1 cotransporter keeps the Cl− reversal potential above resting membrane potential. On the other hand, the KCC2 cotransporter, which extrudes Cl− from the cell, is expressed as development progresses, countering Cl− accumulation (Ludwig et al. 2003; Rivera et al. 1999; Yamada et al. 2004). Interestingly, however, NKCC1, which is initially expressed in somata, does not simply disappear but is redistributed toward the dendritic region during the first three postnatal weeks (Marty et al. 2002), suggesting the depolarizing effects of GABA in dendrites, while already hyperpolarizing the soma and basal dendrites. We tested this hypothesis by a number of different experiments involving focal application of muscimol, a specific GABA_A-R agonist, evolving postsynaptic potentials by direct activation of interneurons in stratum radiatum (SR) and stratum oriens (SO) and, finally, by exploiting the fact that dentate gyrus (DG) stimulation can induce monosynaptic GABAergic potentials in CA3 during development (Gutiérrez et al. 2003; Kasyanov et al. 2004; Safiulina et al. 2006; Walker et al. 2001), in the presence of glutamate receptor antagonists. We recorded in area CA3c, where mossy fibers (MFs) innervate both basal and apical dendrites of CA3 pyramidal cells (Blackstad et al. 1970; Claiborne et al. 1986). This also permitted us to analyze field potentials and construct current source density (CSD) profiles in the presence of glutamate receptor antagonists.

Measuring the depolarizing or hyperpolarizing activity of GABA is not easy because cell dialysis complicates E_GABA determination with patch and sharp microelectrodes. On the other hand, assessing the excitatory actions of GABA by testing for modulation of epileptiform activity, or after changing [K+], has the disadvantage of altering the driving force for K+−Cl− cotransport (Müller et al. 1989; Thompson and Gählwiler 1989). These problems can be circumvented by the use of recordings of GABA-R–mediated field potentials (IPSPs) (Treviño and Gutiérrez 2005). These are negative when GABA causes depolarization due to Cl− flow out of the cells and positive when Cl− enters the cells, thereby causing hyperpolarization. The analysis of field potentials mediated by GABA-R activation and subsequent CSD analysis indicate a delayed GABA shift in the dendritic compartment whereby GABA exerts locally depolarizing actions until postnatal day (P)18. We suggest that GABA-R–mediated responses in PyrC
are compartmentalized throughout development, whereby GABA still exerts depolarizing effects in the dendritic compartment when GABA already produces hyperpolarizing responses in the somatic compartment.

**METHODS**

Adult and young Wistar rats at different postnatal ages were used in our experiments (see RESULTS). Some experiments were also performed on slices from pentyleneetetrazol (PTZ; 60 mg/kg, administered intraperitoneally) injected adult rats that had suffered a convulsive seizure. All experimental procedures were approved by the Ethics Committee for Animal Research at our institution. The rats were decapitated under deep ether anesthesia, their brains were rapidly removed, and combined entorhinal cortex–hippocampus slices (400 μm) were prepared in oxygenated artificial cerebrospinal fluid (ACSF) at 4°C as described previously (Gutiérrez 2000). For recordings, the preparations were placed in a liquid-to-air interface recording chamber and perfused constantly (1 ml/min) with ACSF containing (in mM): NaCl, 129; KCl, 3; NaH2PO4, 1.25; MgSO4, 1.8; CaCl2, 1.6 or 0; NaHCO3, 20; and glucose 10 (pH 7.35); or with a carbonate-free ACSF containing NaCl, 150; KCl, 2.5; NaHPO4, 1.3; MgCl2, 2; CaCl2, 2; HEPES, 10; and glucose 10 (pH 7.35), at 34°C. We recorded (AxoClamp 2B amplifier; Axon Instruments) extracellular and intracellular responses from the CA3b-c area to stimulation of the molecular layer of the dentate gyrus (DG: pulse duration 0.1 ms at 0.05 Hz) applied with bipolar glass-insulated platinum wire electrodes (25 μm), and intracellular responses to stimulation of SO and SR of area CA3 applied with ACSF-filled patch pipettes (5 MΩ). We used borosilicate microelectrodes of 8–12 MΩ filled with NaCl (155 mM) for extracellular recordings and of 70–90 MΩ filled with potassium acetate (2 M) for intracellular recordings. It is often argued that sharp microelectrodes lead to alterations of intracellular potassium and chloride concentration due to leakage. We determined K+ release from intracellular recording electrodes with resistances of >80 MΩ by use of a K+-sensitive microelectrode in close proximity to the ion-sensitive microelectrode (15 μm in the extracellular space and 1–5 μm in ACSF). There were no measurable elevations in K+ concentration even when currents of 1 nA were applied. Leak of K+ was noted only when electrodes were broken back to resistances of <15 MΩ. Ionophoresis of K+ by positive current injection with current intensities of >100 nA were required to elevate K+ concentration by >2 mEq (see Lux and Neher 1973; Pässler et al. 2007). We conclude that recordings with sharp microelectrodes will not significantly alter intracellular Cl− and K+ concentration.

In each experiment, we determined the input/output curves and the stimulation intensity was fixed at a value that evoked 60% of the maximal fIPSP during control ACSF perfusion (Gutiérrez 2000). Additionally, GABA_{A}-mediated responses were evoked by focal application (air puff pulses of 20 ms; 35–50 psi) into the SR or stratum pyramidale (SP) of the GABA_{A}-agonist muscimol (1 mM diluted in ACSF) with a patch pipette (10 MΩ). To evoke monosynaptic responses in the distal and basal dendrites of pyramidal cells, monopolar pulses of 0.1 ms were applied through a glass pipette (1–2 MΩ) filled with ACSF and placed in the SR and SO, respectively, in the presence of ionotropic glutamate receptor (iGluR) antagonists. All drugs used were diluted in ACSF including: the N-methyl-D-aspartate receptor (NMDA-R) antagonist (30 μM), 2-amino-5-phosphonovaleric acid (APV, 30 μM; Tocris Cookson; Ballwin, MO); the non-NMDA-R antagonist 6-nitro-7-sulamoylbenz(quinolinoxaline-2,3-dione (NBQX, 10 μM; Tocris Cookson); the GABA_{A}-R antagonists bicuculline methiodide (20 μM; Sigma) and SR95531 (gabazine; 20 μM; Sigma); the GABA_{B}-R antagonist 2S-3, [1S]-1-(3,4-dichlorophenyl)ethylamino-2-hydroxypropyl)[phenylmethyl]phosphonic acid (CGP55845A, 1 μM; a gift from Novartis); the group III metabotropic glutamate receptor (mGluR) agonist L-AP4 (10 μM; Tocris Cookson); the cation–chloride cotransporter blockers bumetanide (10 μM in 10% DMSO; Tocris Cookson) and furosemide (500 μM; Sigma); and the membranal GABA transporter 1 (GAT-1) blocker nipecotic acid (10 μM; Sigma).

The afferent volley was isolated at the end of the experiments in which extracellular recordings were obtained by the simultaneous perfusion of GluR antagonists and bicuculline, or of Ca^{2+}-free ACSF. The electrophysiological signals were acquired with pClamp8 software (Axon Instruments, Foster City, CA) and the extracellular recordings were exported and analyzed with a program written in MATLAB 7.0-R14 (The MathWorks, Natick, MA). This program computes the averages of sets of six successive fIPSPs throughout the experiment and then carries out the point-to-point subtraction of the synaptic responses obtained under different pharmacological conditions (see Treviño and Gutiérrez 2005). The effect of L-AP4 on the isolated GABAergic response was tested routinely prior to bicuculline perfusion to confirm the origin of the GABAergic response. One-dimensional current source density analysis (CSDA) was carried out in 20 slices from three 6-day-old rats, in 20 slices from four 15-day-old rats, and in 12 slices from six 22-day-old rats, in control ACSF, during perfusion of GluR antagonists, and during perfusion of GluR + GABA_{A}-R antagonists. Field responses (averages of 10) to DG activation were recorded sequentially along the pyramidal cell axis, from stratum oriens to stratum radiatum at 20-μm intervals (Δx). The depth of the recording electrode was adjusted to maximize the peak amplitude of the recorded field potential (usually 150–250 μV under the slice surface). To obtain the glutamatergic currents, the currents calculated during perfusion of GluR blockers were subtracted point to point from those obtained in control ACSF. For the isolation of the GABAergic currents, the currents calculated during perfusion of GluR blockers and bicuculline were subtracted point to point from those obtained during perfusion of GluR antagonists, as previously described (Treviño and Gutiérrez 2005). This procedure is a valid means to isolate the pharmacologically treated currents as the recorded extracellular current density represents the algebraic sum of all currents (Kwan and Murphy 1974; Mitzdorf 1985). Ten different experiments of each experimental group were averaged using an area-based registration algorithm. Using the Fourier–Mellin transform, this function optimizes x/y translations to maximize the correlation between contours and then computes the CSD average ± SE whenever contours overlap (Kuglin and Hines 1975). Stimulus artifacts in the CSD maps were suppressed for clarity. Depth profiles were computed at max [CSD(x, y)] ± SE from the averaged CSD contours. The significance of differences between groups (P < 0.05) was assessed using paired Student t-test.

**RESULTS**

**Effect of direct activation of distal dendritic and somatic GABA_{A}-R with muscimol during development**

Because activation of GABA_{A}-Rs before P9 causes depolarization, we conducted puff application of muscimol for ∼20 ms. At P6 muscimol application to both the apical dendrite and the soma level caused depolarization associated at each site with a negative field potential (n = 3). To address the possibility that activation of GABA_{A}-R has differential effects in the somatic and dendritic compartments at P15, when NKCC1 is still expressed in apical dendrites but no longer in the PyR layer (Marty et al. 2002), we analyzed with intracellular and extracellular recordings the responses evoked by the activation of the apical dendritic and somatic GABA_{A}-Rs using pressure-pulse application of the GABA_{A}-R agonist muscimol (1 mM) for 20 ms (Fig. 1A) in the presence of iGluR blockers (NBQX, 10 μM and APV, 30 μM). Intracellular recordings showed that...
somatic application of muscimol with short pressure pulses induces a hyperpolarization. The mean reversal potential for these responses was $-67.2 \pm 0.5$ mV ($n = 3$) as determined by application of muscimol at different membrane potentials using hyperpolarizing current injection. The extracellular responses recorded in SR showed a clear low-amplitude positive deflection. In contrast, the distal dendritic application of muscimol produced a depolarization of the membrane potential that did not reverse on depolarizing current injection to different membrane potentials. Linear extrapolation yielded an estimate of $-62.5 \pm 0.5$ mV for the reversal potential ($n = 3$). The extracellular recordings in s. lucidum/radiatum (SL/R) showed a negative deflection (Fig. 1, B and C). As expected, the intracellular and extracellular responses evoked by muscimol were blocked by bicuculline (Fig. 1, B and C). Because GABA can cause depolarization through a HCO$_3^-$-mediated current (Kaila et al. 1997), we repeated the experiments in HCO$_3^-$-free ACSF in 6- and 15-day-old preparations, and in adult preparations from PTZ-treated rats ($n = 3$ at each age). Under these conditions, application of muscimol to apical dendrites could still evoke negative field potentials similar to those evoked in the presence of HCO$_3^-$ that could be blocked by bicuculline, showing that the flux of HCO$_3^-$ is not responsible for the depolarizing actions of GABA in these age groups (Fig. 1C). These results together with the intracellular recordings in PyrC suggest that the muscimol-induced GABA$_A$-mediated negative iPSPs are due to Cl$^-$ outflow from cells resulting in depolarization of pyramidal cells at P6 and depolarization in apical dendrites still at P15.

**Synaptic activation of distal dendritic GABA$_A$-R depolarizes, whereas activation of proximal receptors hyperpolarizes, pyramidal cells at P15**

We next decided to directly stimulate interneurons that activate either distal dendritic or proximal GABA$_A$-R by local stimulation in the SR or in the SO, respectively, in the presence of iGluR and GABA$_B$ blockers (Davies et al. 1993). Intracellular recordings were obtained from 30 PyrCs at P15 that had a resting membrane potential (RMP) of $-64 \pm 0.2$ mV. The responses evoked by SO-Int stimulation (Fig. 2A$_1$) reversed at $-76 \pm 1$ mV, had a 10–90% slope of $-417.5 \pm 37 \mu$V/ms with a time to peak of $10.8 \pm 1.1$ ms ($n = 3$, Fig. 2A$_1$). In contrast, the synaptic responses evoked by SR-Int stimulation could not be reversed, but the estimated reversal potential determined by linear extrapolation was $-59.7 \pm 1.2$ mV with a slope of $45.4 \pm 4.7 \mu$V/ms and a time to peak of $26.3 \pm 5.3$ ms (Fig. 2A$_2$, $n = 19$). PyrC recordings from preparations of adult rats showed RMP of $-63.6 \pm 0.6$ mV ($n = 7$), which was not statistically different from the RMP observed in PyrC of P15 animals ($P < 0.05$). In these preparations SR-Int stimulation provoked hyperpolarizing responses that were blocked by gabazine. The estimated reversal potential for these responses was $-69.6 \pm 1.5$ mV, with a slope of $-136.7 \pm 28.3 \mu$V/ms and a time to peak of $21.1 \pm 2.2$ ms (Fig. 2B, $n = 7$). A summary of the
The polarity of the synaptic dendritic responses obtained during development is suggestive of an outward Cl⁻ current. Therefore we tested the effects of blocking K⁺–Cl⁻ cotransport mediated by NKCC1 with bumetanide (10 µM) (Dzhala et al. 2005; Payne et al. 2003) on the intracellular GABA<sub> Każdy</sub>–mediated responses evoked by distal and proximal synaptic activation at P15. Bumetanide significantly decreased the peak amplitude of the responses to SR-Int stimulation by 74 ± 3% (Fig. 2C). The GABA<sub>waukee</sub>–mediated dendritic responses to SR-Int stimulation were estimated to reverse at −64.2 ± 0.5 mV (n = 4), 5 mV more negative than that observed in the absence of bumetanide (Fig. 3F). As expected, in adult prep-

FIG. 2. Responses evoked by synaptic activation of distal dendritic and proximal GABA<sub>waukee</sub>Rs at postnatal day (P)15. A<sub>1</sub> depicts the sites of stimulation to evoke the responses shown in A<sub>2</sub> and A<sub>3</sub>; and B<sub>1</sub> shows the sites of stimulation to evoke the responses shown in B<sub>2</sub>. A<sub>2</sub>: activation of proximal GABA-R (probably in basal dendrites) by stimulation of interneurons of the stratum oriens (SO-Int) produces hyperpolarizing responses in a PyrC. By contrast, activation of stratum radiatum interneurons (SR-Int) provokes depolarizing responses by activating distal dendritic GABA-R. The responses are blocked by gabazine. The depolarizing GABA<sub>waukee</sub>–mediated responses to SR-Int activation at different membrane potentials are consistent with a depolarized E<sub>Cl</sub>. All traces depicted in A<sub>2</sub> and A<sub>3</sub> correspond to a single PyrC. B<sub>1</sub>: by contrast, synaptic activation of distal dendritic GABA-Rs provokes gabazine-sensitive hyperpolarizing responses in adult preparations. The gray lines indicate the estimated reversal potential of the GABA-R–mediated responses. C: the depolarizing responses to SR-Int activation are strongly depressed by bumetanide but not affected by L-(−)-2-amino-4-phosphonobutyric acid (L-AP4). D: in adult preparations, SR-Int stimulation provokes gabazine-sensitive hyperpolarizing responses, which are insensitive to bumetanide.
Differential shift of GABA action in pyramidal cells

After establishing the differential effects of the activation of distal and proximal GABA<sub>A</sub>-R during development, we sought to explore the effects of the activation of the natural input pathway to CA3, the MF, on its intracellular and field responses. We first corroborated that DG stimulation produced depolarizing potentials during the first week of age and hyperpolarizing responses from the second week of age in the presence of glutamate-R antagonists NBQX (10 μM) and APV (30 μM). The intracellular recordings confirmed that the apparent reversal potential of the synaptically evoked IPSPs in 6-day-old rats was significantly more depolarized (IPSP<sub>rev</sub> = −47 ± 5 mV) than the resting membrane potential (RMP = −64 ± 2 mV; n = 9; t-test: P < 0.05; Fig. 3A), whereas at P15 the IPSP<sub>rev</sub> was significantly more hyperpolarized (RMP = −62 ± 2 mV; IPSP<sub>rev</sub> = −68 ± 1 mV; n = 30; t-test: P < 0.05; Fig. 3B). IPSPs recorded were completely blocked by the addition of the GABA<sub>A</sub>-R antagonists bicuculline (20 μM) or gabazine (Fig. 3C). By contrast, PyrC from adult rats had RMP of −63.6 ± 0.6 mV but did not present synaptic responses by dentate stimulation in the presence of glutamatergic blockers (Fig. 3D). As expected for the activation of proximal GABA-R at P15, bicuculline had no effect on DG-evoked responses (Fig. 3E). Finally, to corroborate the origin of the evoked responses, we tested the effect of the mGluR agonist L-AP4 (20 μM), which selectively depresses responses of MF origin (Gutiérrez 2000, 2002; Safiulina et al. 2006). In the presence of L-AP4, peak amplitudes of the DG responses were markedly depressed (Fig. 3E). Noteworthy, the stimulation of the DG-evoked responses in P15 preparations had a reversal potential value between those obtained for SO and SR stimulation, as expected if MFs release GABA on both the apical and basal dendrite (Fig. 3F).

GABA<sub>A</sub>-R-mediated synaptic field potentials in developing rats elicited by DG stimulation

Because synaptically driven GABA-R-mediated responses can be recorded extracellularly (Treviso and Gutiérrez 2005), we hypothesized that conducting such recordings at different sites could reveal the spatial- and time-dependent shift of GABA actions from depolarization to hyperpolarization during development. DG stimulation, which evoked an intracellular depolarizing response in P6 PyrCs, evoked a negative (downward) IPSP in the SL (Fig. 4A). The perfusion of NBQX and APV did not completely isolate the afferent volley, suggesting the presence of an underlying GABAergic component. The additional perfusion of the GABA<sub>A</sub>-R antagonist bicuculline or gabazine led to total suppression of the remaining synaptic

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**Fig. 3.** Age-dependent shift in polarity of dendritic and somatic GABA-R-mediated responses. Intracellular recordings of GABA<sub>A</sub>-R-mediated DG-evoked inhibitory postsynaptic potentials (IPSPs) at different membrane potentials in a P6 (A) and a P15 CA3 PyrC (B), in the presence of iGluR blockers. At P6, the IPSP reverses at a more depolarized value than the RMP, whereas at P15 the IPSP reverses at a more hyperpolarized value than the RMP. C: gabazine blocks the dentate gyrus (DG)-evoked responses in PyrC. D: by contrast, in adult preparations, DG stimulation does not evoke synaptic responses in the presence of iGluR blockers. E: at P15, the hyperpolarizing responses evoked by DG activation are not affected by bicuculline but are strongly depressed by L-AP4. F: estimated reversal potential, expressed as mean ± SE, of the GABA-R-mediated responses evoked by stimulation of SR-Int, DG, and SO-Int in PyrC in P15 and adult preparations. Reversal potentials of the responses evoked by SR-Int stimulation before (white bars) and after the addition of bicuculline (black bars) were estimated by extrapolation. The gray lines indicate the average RMP. Asterisks signal statistical significant difference at P < 0.005 (Student’s t-test).

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**Legend:**

- **Fig. 3**
- **AB:** 6 days old
- **BD:** 15 days old
- **E:** 15 days old
- **F:** P15

**Table:**

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<tr>
<th>Group</th>
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**Notes:**

- RMP: Resting Membrane Potential
- IPSP<sub>rev</sub>: Reversal Potential of IPSP
- NBQX: N-Butyl-4-carboxy-N-methyl-1-oxoquinoline
- APV: 6-Azetido-2-carboxylic acid
- Bume: Bumetanide
- L-AP4: L-Alpha-Azepan-4-Acetic Acid
component and isolated the afferent volley. By applying a point-to-point subtraction of the responses obtained prior to and after perfusion of the appropriate antagonists, we were able to isolate the GluR-antagonist-sensitive and the GABA-R–antagonist-sensitive components (Treviño and Gutiérrez 2005). As expected, the extracellular synaptic responses obtained in the SL/R had the opposite polarity to the intracellularly recorded postsynaptic potentials (Fig. 4A, n/H11005/H11002 68 mV), whereas the extracellular field response has a negative polarity (thick gray arrow). B: at 15 days of age, the extracellular field potential still has a negative polarity (thick black arrow) despite the shift of the GABA-R–mediated intracellular response from depolarizing to hyperpolarizing (thin black arrow; RMP = −64 mV). C: the extracellular GABA-R–mediated responses shift to positive polarity (thin black arrow) at around P18 and disappear at around P23–P24 (D). From this day on, intracellular (RMP = −66 mV) and extracellular GABA-R–mediated responses can no longer be detected. All traces are averages of 6–10 evoked responses.

FIG. 4. Glutamate-R–mediated and GABA-R–mediated intracellular and extracellular field responses to DG activation can be detected in the CA3 during development. Extracellular field potentials in the SL and intracellular responses from PyrC of CA3 were obtained at different ages under different pharmacological conditions. A: at 6 days of age, the pharmacologically isolated GABA-R–mediated IPSP is depolarizing (thin gray arrow; RMP = −68 mV), whereas the extracellular field response has a negative polarity (thick gray arrow). B: at 15 days of age, the extracellular field potential still has a negative polarity (thick black arrow) despite the shift of the GABA-R–mediated intracellular response from depolarizing to hyperpolarizing (thin black arrow; RMP = −64 mV). C: the extracellular GABA-R–mediated responses shift to positive polarity (thin black arrow) at around P18 and disappear at around P23–P24 (D). From this day on, intracellular (RMP = −66 mV) and extracellular GABA-R–mediated responses can no longer be detected. All traces are averages of 6–10 evoked responses.

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older, the intracellular synaptic responses were completely blocked by the iGluR antagonists and therefore only the glutamate-antagonist-sensitive extracellular component could be isolated (Fig. 4D, n = 25) as reported previously (Gutiérrez et al. 2003). However, from this age on, the intracellular and extracellular GABAergic responses to MF activation could again be recorded in hippocampal slices prepared 2 h after inducing seizures in vivo. As previously described, this leads to upregulation of the GABA synthesizing enzyme, GAD67, GABA itself, and the detection of glutamate- and GABA-mediated monosynaptic responses (not shown; see Treviño and Gutiérrez 2005).

The percentage of rats that presented GABA-R–mediated negative and positive fPSPs at each age is depicted in Fig. 5A. However, in some experiments our subtraction method did not yield detectable extracellular responses because the differential signal was no different from noise. These responses were considered as “not detected” and they are also plotted in Fig. 5A. Isolated GABA-R–mediated responses could not be detected in 66% of the slices from P23 rats (n = 26) and in all rats at P25. We randomly selected 12 experiments of each age group and calculated both the area under the curve of the isolated field synaptic components and the GABA/glutamate-mediated response ratio. Although at P6 the isolated GABAergic component is bigger than the glutamatergic component, it drops to around 45% at P15, P18, and P21 (Fig. 5B), coinciding with the ratio obtained in adult rats subjected to seizures (Treviño and Gutiérrez 2005). In the experiments performed at P23 in which GABA-R–mediated fPSPs were observed, these responses were just above the level of detection. It is noteworthy that the values of the glutamatergic component are possibly underestimated because DG stimulation in control ACSF monosynaptically activates both PyrC and interneurons, whose activation also contributes to the fPSP. Thus the GABAergic-to-glutamatergic ratio in pyramidal cells could be greater than that reported here.

As previously shown, MF-GABAergic intracellular and fPSPs from CA3 are preferentially depressed by the activation of cation–Cl cotransport. Bumetanide blocked the negative field responses evoked at P6 and P15, but not the positive responses evoked at P21. E: at P15, the blockade of GAT-1 with nipecotic acid potentiates the GABA-R–mediated responses (n = 6). F: bar graph depicting the effects of the cation–Cl cotransport blockers bumetanide and furosemide and of the GAT-1 blocker on the GABA-R–mediated field responses of the whole series of experiments. Note that the cation–Cl cotransport blockers are not effective when the field potentials have shifted to a negative polarity at P21, whereas the GAT-1 blocker strongly potentiates the GABA-mediated field responses.

**FIG. 5.** Age-dependent shift in polarity of the synaptically induced extracellular GABA-R–mediated dendritic currents evoked in s. lucidum (SL) by DG activation. A: extracellular potentials have a negative polarity during the first two and a half weeks of life. They progressively shift in polarity from negative to positive at P18 until they are virtually only positive at around P21. At P23–P24, both intracellular and extracellular GABA-R–mediated responses disappear. Squares linked by a line represent the percentage of experiments in which the GABAergic component could not be detected (% n.d.) with our subtraction method. B: bar graph depicting the relative weight of the GABAergic component in the field responses at different ages when compared with the glutamatergic component (% cases) and calculated both the area under the curve of the isolated field synaptic components and the GABA/glutamatergic ratio. Although at P6 the isolated GABAergic component is bigger than the glutamatergic component, it drops to around 45% at P15, P18, and P21 (Fig. 5B), coinciding with the ratio obtained in adult rats subjected to seizures (Treviño and Gutiérrez 2005). In the experiments performed at P23 in which GABA-R–mediated fPSPs were observed, these responses were just above the level of detection. It is noteworthy that the values of the glutamatergic component are possibly underestimated because DG stimulation in control ACSF monosynaptically activates both PyrC and interneurons, whose activation also contributes to the fPSP. Thus the GABAergic-to-glutamatergic ratio in pyramidal cells could be greater than that reported here.
of the group III mGluR (Gutiérrez 2002, 2005; Gutiérrez et al. 2003; Kasyanov et al. 2004; Treviño and Gutiérrez 2005). Thus we routinely tested the effect of L-AP4 (10 μM) on the bicuculline-sensitive DG-evoked fPSPs recorded in the SL/R in the presence of NBQX plus APV. As expected, L-AP4 reversibly inhibited the GABA-mediated fPSPs by 82.8 ± 2.9% (n = 15; Fig. 5C).

GABA<sub>A</sub>-mediated population responses are prevented by blockage of the outward transport of Cl<sup>−</sup> and they are potentiated by blocking GAT-1

The polarity of the synaptic dendritic responses obtained during development is suggestive of an outward Cl<sup>−</sup> current. Therefore we tested the effects of blocking K<sup>+</sup>–Cl<sup>−</sup> cotransport mediated by NKCC1 with furosemide (500 μM) and bumetanide (10 μM) (Dzhala et al. 2005; Payne et al. 2003). As expected, the negative field potentials in SR induced by DG stimulation at P6 (n = 6) and P15 (n = 10) were blocked by the perfusion of both cotransporter antagonists (Fig. 5, D and F). However, when the field GABA-R–mediated responses shifted direction at P21, these blockers were ineffective (Fig. 5, D and F; n = 6).

Because antagonists of the GAT-1 membrane transporter increase GABA-R–mediated spontaneous and evoked activity in CA3 (Sipilä et al. 2004; Vivar and Gutiérrez 2005), we examined how blocking GAT-1 affected the pharmacologically isolated GABA-R–mediated negative field response in SL at P15. The perfusion of nipeotic acid (10 μM) reversibly augmented the area under the curve of the fPSP by 313 ± 45% (n = 6; Fig. 5, E and F).

**Current source density analysis**

Because activation of remote areas may lead to far-field effects, the recorded field potentials may not show the local current flows. In addition, current flow from horizontally oriented elements may contribute to the field potentials. We therefore performed a current source density analysis (CSDA) along the orientation of the apical dendrites, which removes far-field effects and shows where currents enter and leave the extracellular space. Our subtraction approach can also determine the direction of the studied GABAergic synaptic currents. Therefore, we performed one-dimensional CSDA before and after blockade of ionotropic GluR and GABA-R in slices from 6-, 15-, and ≥22-day-old rats (3/group age), as previously described (Treviño and Gutiérrez 2005). In slices from 6-day-old rats the point-to-point digital subtraction of the CSDA calculated in normal ACSF from that in the presence of GluR antagonists isolated a glutamatergic current sink in the SL/R and also in the SO of CA3 (not shown). Furthermore, the point-to-point subtraction of the CSDA calculated in the presence of GluR and GABA-R antagonists from that obtained in the sole presence of GluR antagonists also yielded a current sink in the same regions (Fig. 6, A and B). At P15 there was an overlap of the current sinks mediated by glutamatergic and of the current sources mediated by GABAergic synaptic transmission in SO (Fig. 6, B and C), whereas in SR an extended

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**FIG. 6.** High-resolution current source density analysis (CSDA) averaged profiles, presented in the form of contours, were computed for the isolated glutamatergic and GABAergic signals in preparations at P6 (A; n = 14) and P15 day (B; n = 12). The glutamatergic component was computed by point-to-point digital subtraction of the contour maps obtained before and after perfusion of GluR antagonists, and the GABAergic component by subtraction of the maps obtained before and after adding GABA<sub>A</sub>-R antagonist. Both, the Glu-R and the GABA-R–mediated dendritic responses produce current sinks in the dendritic region at P6. This current sink was also produced by the glutamatergic component. However, at P15, the same stimulation produced a current source in the SL region, which is indicative that the GABA<sub>A</sub>-R–mediated responses produce outward currents. Note the temporal and spatial coincidence of the GABAergic current sinks and sources in the averaged depth profiles. Asterisks signal the 1.5-ms window at which the peak currents were detected to construct the depth profiles in 9 P6 and 8 P15 rats.
GABAergic sink was still present. These results, depicted in the averaged depth profiles, are consistent with GABA-R–mediated outward currents in SO carried by Cl− inward movements (Fig. 6C). In contrast, the GABAergic component observed in SO had a polarity different from that recorded in SL and was observed as a current source (Fig. 6C).

**Discussion**

We show with extracellular recording of dendritic current flow evoked by GABA-R activation at P15 that a depolarizing current coexisted with intracellularly recorded hyperpolarizing IPSPs. Thus our results are in agreement with the idea that in dendrites the shift in the action of GABA from depolarization to hyperpolarization is delayed to the third postnatal week, provoking GABAergic inputs to produce outward Cl− currents, whereas in somata this reversal occurs from the beginning of the second week. Our conclusion is supported by local activation of distal and proximal GABA-R with muscimol and also by measurements of responses to isolated activation of interneurons in SR and SO of area CA3. Moreover, taking advantage of the putative GABAergic cotransmission from MF terminals during development, we show that in the presence of iGluR blockers, bulk stimulation of the DG produces GABA-R–mediated IPSPs in the SL/R of CA3 that disappear by the end of the third postnatal week. Therefore our results unravel a functional role of the redistribution of the NKCC1 cotransporter from the somata toward the dendritic region during the first three postnatal weeks (Marty et al. 2002). It is worth mentioning that the antibody used in the study by Marty et al. (2002) is known to recognize NKCC1 and -2 isoforms in the brain.

Direct activation of distal dendritic and proximal GABA receptors

It has long been recognized that activation of GABAARs can produce biphasic hyperpolarizing–depolarizing responses in pyramidal cells (Alger and Nicoll 1979) and that activation of distal dendritic receptors can depolarize the cell to produce inhibition by a shunting effect (Andersen et al. 1980). In pyramidal cells of the cortex, it was shown that direct application of GABA had differential effects on the dendritic and the somatic compartments during development (Luhmann and Prince 1991).

Relatively long iontophoretic application of GABA to dendrites of PyrCs produces outward Cl− currents, whereas somatic GABA-R activation produces inward Cl− currents (Jarolimek et al. 1999), potentially resulting from a strong contribution of HCO3− to dendritic responses when Cl− reversal potential is clamped to resting membrane potential. We show here that application of the GABAAR agonist muscimol applied for very short times, thereby inducing local activation of dendritic GABAAR, is reflected as a depolarization in intracellular recordings and produces extracellular field potentials with the same polarity as that evoked by DG activation following blockade of iGluR. In our recordings we used application times for muscimol of 20 ms. In thus preventing prolonged effects of GABA-R activation, which can result in a shift from hyperpolarizing to depolarizing due to the contribution of bicarbonate to GABA-mediated responses (Kaila et al. 1997). We nevertheless checked whether the depolarizing responses were dependent on HCO3− (Burg et al. 1998) and found that they were not.

Effects of direct activation of interneurons by local stimulation

One might argue that the DG-evoked IPSPs recorded in SL derive not from MF synaptic input onto PyrCs but instead from responses of inhibitory interneurons. If this were the case, their local activation would result in extracellular and intracellular responses indicative of hyperpolarization at both the soma and the dendrites. However, the stimulation of SR-Int in the presence of iGluR antagonists evoked a depolarizing response due to synaptic activation of distal GABAAR, whereas stimulating interneurons in the SO resulted in hyperpolarization of CA3 PyrCs following P10. This fact suggests that proximal GABAergic inputs dominate the somatic responses evoked by MF stimulation and that the IPSPev, which is more depolarized in response to DG activation than in response to SO-Int stimulation, reflects the summed effects of both basal and apical dendritic input. Indeed, in CA3c, where our recordings were done, the PyrCs receive a MF projection not only to the apical dendrite but also to the basal dendrites (Blackstad et al. 1970; Claiborne et al. 1986). Also, in the case of SR-Int stimulation, blockade of NKCC1 cotransporter blocks depolarizing PSPs and extracellular responses, which is in line with the view that NKCC1 maintains elevated Cl− concentrations in apical dendrites from PyrCs (Marty et al. 2002). Bumetanide has a high affinity for NKCC1 at the concentration used without significantly affecting KCC2 (Payne et al. 2003). Since bumetanide suppresses sharp waves and giant depolarizing potentials in developing CA3 (Sipilä et al. 2006), NKCC1 appears to underlie both phenomena.

Responses to DG stimulation

The excitatory and inhibitory actions of GABA during development have mainly been studied by observing the effects of focal applications to the dendrites or soma during evoked epileptiform activity or after changing [K+]o, usually with whole cell recordings. However, these strategies alter the driving force of the K+−Cl− cotransport (Müller et al. 1989; Thompson and Gähwiler 1989). These problems can potentially be circumvented by analyzing extracellular recordings that detect synthetically evoked GABA-R–mediated currents in a synapse that transiently releases both glutamate and GABA during development (Gutiérrez et al. 2003; Safiulina et al. 2006; Walker et al. 2001). We have previously shown that a positive IPSP can be evoked in SL in slices of adult rats after seizures, in the presence of iGluR blockers (Trevisio and Gutiérrez 2005); in contrast, we now observe in the same layer a negative t-AP4–sensitive fIPSP during perfusion of iGluR antagonists that persists until P18. This potential component is blocked by bicuculline and gabazine and it is augmented by blockade of GAT1, the GABA membrane transporter that removes GABA from the extracellular space and prolongs DG-evoked iPSPs (Vivar and Gutiérrez 2005). Before P9 this fIPSP is associated with a depolarizing GABA-R–mediated PSP in intracellular recordings. After P10 and until P18, however, when intracellular responses were already hyperpolarizing, the
negative dendritic fPSP persisted, suggesting an outward Cl⁻ flux in apical dendrites.

It is worth noting that bumetanide virtually completely blocked the extracellular responses in SL/SR at P15, whereas the intracellular responses were strongly reduced. This difference can be accounted for by the fact that measurements of the isolated GABA-R–mediated extracellular response is conducted in the SL/SR region, where the responses of hundreds of cells contribute to the population potential, probably masking the variability of single cell responses. In general the extracellular responses are more sensitive to block than intracellular signals because the extracellular signals depend on summation of activities in many neurons that show up as field potentials only when the cells respond with very similar latencies.

Extracellular potentials may arise through activation of interneurons and in some cases glia, and the positive fPSPs in dendritic fields during application of muscimol and during stimulation of MFs could result from activation of interneurons, in which the shift from depolarizing to hyperpolarizing actions of GABA might occur later during development. In our experiments, polysynaptic interneuronal inhibition is blocked by GluR antagonists when GABA-R–mediated currents are resolved. Also, GABA-R–mediated activation of interneurons is unlikely because GABA has an inhibitory effect on interneurons during postnatal development (Banke and McBain 2006). Thus the detection of GABA-R–mediated fPSPs is possible only if a considerable amount of MF boutons simultaneously release GABA at a restricted site that should act on a large number of postsynaptic receptors in CA3 PyrCs. These findings are also supported by the current source density analysis, which removes far-field effects. This analysis revealed spatially restricted current sink at the same location, with intact glutamatergic synaptic activity and, after it is blocked, as expected when MFs corelease GABA and glutamate. The glutamatergic sink at the same site is opposed by a GABAergic source at the identical site when corelease of GABA has been reintroduced due to upregulation of markers of the GABAergic phenotype in GCs and their MFs (see Gutiérrez 2005). Moreover, we observed that at P15, DG electrical stimulation generates a GABA current source in SL, consistent with a hyperpolarizing somatic GABA response. Furthermore, a second active GABA source at the level of the basal dendrites may also contribute to the hyperpolarizing nature of the DG stimulation-induced GABAergic (somatic) response at this age. However, when comparing the sink source distribution between P6 and P15 we note already at P6 a spatially extended sink in SL/SR, which is now replaced by a source close to SP, and an extended sink in SL/SR, which is larger than the accompanying source between SP and SR. It is well possible that the field potential is negative at the transition between these sinks and sources. The finding may indicate that a distributed release of GABA along the apical dendrite may induce a Cl⁻–driven inward current close to the soma and a Cl⁻–driven outward current at more apical dendrites. The differences in reversal potentials between the different stimulation sites are in keeping with this conclusion. By taking this information together, it would appear likely that the DG-activated GABA inputs into CA3 pyramidal cells cover a broad dendritic range along which the GABAergic responses turn from hyperpolarizing (proximal) to depolarizing (distal).

**Physiological implications**

Our results provide a functional role of the redistribution of the NKCC1 cotransporter from the somata toward the dendritic region during the first three postnatal weeks (Marty et al. 2002). Although the chloride gradient breaks down faster in the dendrite than in the soma, it is likely that the action of NKCC1, which is relocalized to the dendritic region, maintains the chloride uptake active thus rendering GABA depolarizing (Achilles et al. 2007).

Even when the effects of GABA change from depolarizing to hyperpolarizing, excitability and epileptogenesis are still high in juvenile rats (Dzhala and Staley 2003; Gloveli et al. 1995). There is a strong correlation between the postnatal age at which GABAₐ₋ₐ antagonists decreased action potential frequency and the age at which ictal activity can be induced by elevated potassium (Dzhala and Staley 2003). However, it was not clear why GABA had excitatory effects, particularly considering that GABA intracellular responses are hyperpolarizing from day 10. In the rat hippocampus, the change in polarity of the extracellular GABA-R–mediated SL responses signals the end of the excitatory and pro-epileptogenic nature of GABA by the third week. Indeed, bumetanide exerts antiepileptic effects (Dzhala et al. 2005; Sipilä et al. 2006) at the time we demonstrate that it blocks the MF GABAergic responses producing outward currents in the dendrites. Although the antiepileptic effect of bumetanide depends on how epileptic activity is produced in young hippocampi (Kilb et al. 2007), it is suggestive that in the aforementioned elevated K⁺ model, the time frame of the disappearance of elevated K⁺-induced ictal activity and that of the GABA-mediated SR field responses coincide. Our evidence indicates that compartmentalization of the effects of GABA on PyrCs may underlie the excitatory activity of GABA until the end of the third week, importantly, under physiological conditions, as previously suggested by work in the cortex (Luhmann and Prince 1991). It is noteworthy, however, that although we found that direct or synaptic activation of distal GABAₐ₋ₐ-Rs produced depolarizing responses, in no case did we observe that they reached threshold for provoking action potentials. On the other hand, KCC2, which extrudes Cl⁻ from the cell, is expressed as development progresses, counteracting Cl⁻ accumulation (Ludwig et al. 2003; Rivera et al. 1999; Yamada et al. 2004) and possibly its full expression in CA3 PyrCs (Gulyás et al. 2001) coincides with the corresponding change in the direction of the GABA-R–mediated dendritic currents.

The blockage of the membrane GABA transporter GAT-1 increases the availability of GABA in the extrasynaptic milieu and potentiates GABA-R–mediated IPSPs provoked by MF stimulation (Vivar and Gutiérrez 2005). Moreover, blocking GAT-1 markedly enhances GABAₐ₋ₐ-R–mediated transmission during giant depolarizing potentials, as well as a tonic GABAₐ₋ₐ-mediated current (Sipilä et al. 2007). In this way, GABA from the MFs may contribute to the fine-tuning of the CA3-driven GDPs (Sipilä et al. 2004) to finally model circuit formation and neurite outgrowth (Ben-Ari 2001; Ben-Ari et al. 1994; Owens and Kriegstein 2002).

Finally, by activating the different GABAergic inputs on PyrC, SR, and SO interneurons and the MF, we describe that the intracellularly measured reversal potential of the different GABAergic signals differs consistently. Indeed, the responses
evoked by SR interneuron activation were depolarizing with respect to the RMP, the responses evoked by SO interneuron activation were hyperpolarizing with respect to the RMP, and the responses evoked by MF activation were also hyperpolarizing but to a lesser extent than those evoked by SO interneuron activation. This implies that MF stimulation activates receptors along the proximal apical dendrite as well as receptors located in basal dendrites that, due to different chloride concentrations, cause differential effects in the dendritic and somatic compartments. Importantly, this is also reflected in the field responses, which not only integrate the currents originated in hundreds of cells that have a laminar disposition within the structure, but that also shift in polarity and disappear as development proceeds. This parallels the redistribution and, finally, with the disappearance of NKCC1 and expression of KCC2.

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