

# Glutamate Inhibits GABA Excitatory Activity in Developing Neurons

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In contrast to the mature brain, in which GABA is the major inhibitory neurotransmitter, in the developing brain GABA can be excitatory, leading to depolarization, increased cytoplasmic calcium, and action potentials. We find in developing hypothalamic neurons that glutamate can inhibit the excitatory actions of GABA, as revealed with fura-2 digital imaging and whole-cell recording in cultures and brain slices. Several mechanisms for the inhibitory role of glutamate were identified. Glutamate reduced the amplitude of the cytoplasmic calcium rise evoked by GABA, in part by activation of group II metabotropic glutamate receptors (mGluRs). Presynaptically, activation of the group III mGluRs caused a striking inhibition of GABA release in early stages of synapse formation. Similar inhibitory actions of the group III mGluR agonist L-AP4 on depolarizing GABA activity were found in developing hypothalamic, cortical, and spinal cord neurons *in vitro*, suggesting this may be a widespread mechanism of inhibition in neurons throughout the developing brain. Antagonists of group III mGluRs increased GABA activity,

suggesting an ongoing spontaneous glutamate-mediated inhibition of excitatory GABA actions in developing neurons. Northern blots revealed that many mGluRs were expressed early in brain development, including times of synaptogenesis. Together these data suggest that in developing neurons glutamate can inhibit the excitatory actions of GABA at both pre-synaptic and postsynaptic sites, and this may be one set of mechanisms whereby the actions of two excitatory transmitters, GABA and glutamate, do not lead to runaway excitation in the developing brain. In addition to its independent excitatory role that has been the subject of much attention, our data suggest that glutamate may also play an inhibitory role in modulating the calcium-elevating actions of GABA that may affect neuronal migration, synapse formation, neurite outgrowth, and growth cone guidance during early brain development.

**Key words:** *hypothalamus; metabotropic glutamate receptor; calcium; developing synapse; spinal cord; cortex*

During development, the primary inhibitory transmitter of the mature brain, GABA, assumes an excitatory role. Because of an elevated  $\text{Cl}^-$  reversal potential found in immature hypothalamic neurons, activation of the  $\text{GABA}_A$  receptor leads to an inward current caused by  $\text{Cl}^-$  efflux, membrane depolarization,  $\text{Ca}^{2+}$  influx associated with the activation of voltage-activated calcium channels, and an increase in action potentials (Chen et al., 1996). The excitatory actions of GABA are found not only in hypothalamic neurons, the focus of the present study, but also in the majority of developing neurons from all other brain regions we and others have studied, including hippocampus, olfactory bulb, spinal cord, striatum, cerebellum, and cortex (Connor et al., 1987; Cherubini et al., 1990, 1991; Yuste and Katz, 1991; Ben Ari et al., 1994; Reichling et al., 1994; LoTurco et al., 1995; Obrietan and van den Pol, 1995; Serafini et al., 1995; Chen et al., 1996). During development, GABA can influence neurite outgrowth, branching, synapse formation, cell division, and gene expression (Spoerri, 1988; Michler, 1990; Barbin et al., 1993; LoTurco et al., 1995); many of these effects of GABA may be caused by its depolarizing and calcium-elevating actions.

Even at the earliest embryonic times examined in detail [embryonic day 15 (E15)], all hypothalamic neurons expressed func-

tional  $\text{GABA}_A$  receptors. Most neurons also expressed glutamate receptors within the next 3 d (Chen et al., 1995; van den Pol et al., 1995). Studies in the hypothalamus (Chen et al., 1995) and other brain regions (Reynolds and Brien, 1992; Ben-Ari et al., 1994) suggest that GABAergic activity develops early and that glutamate activity occurs soon after. This raises the question as to the possible interaction between the two primary transmitters of the brain during early development. If both GABA and glutamate are excitatory, what prevents the neurons in the developing brain from simply getting caught in a positive feedback cycle of runaway excitation that might lead to seizure-like activity and potentially raise intracellular calcium to cytotoxic levels? At a more local cellular level, can glutamate, rather than summing with GABA to increase excitation and intracellular calcium, act to reduce GABA actions, and, thereby, play an important modulatory role in inhibiting excitation at developing GABAergic synapses?

In this paper we test the hypothesis that inhibitory actions of glutamate in developing hypothalamic neurons reduce the excitatory activity of GABA. We used the hypothalamus because of the relatively large proportion of GABAergic cells and presynaptic boutons (Decavel and van den Pol, 1990). Whole-cell patch clamp recording was used to assess fast ionic currents and potentials in cultured neurons and hypothalamic slices. Because cytosolic calcium levels may play an important role in early development, including regulation of transmitter release, gene expression, neuronal migration, programmed cell death, and extension and turning of growing neurites, fura-2 digital calcium imaging was used to examine cytoplasmic calcium responses. Northern blots were used to test the hypothesis that metabotropic

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glutamate receptors that may modulate GABA activity were expressed very early in brain development. We identify several distinct mechanisms that can account for glutamate-mediated inhibition of GABA excitation in early neuronal development.

## MATERIALS AND METHODS

**Tissue culture.** Hypothalami were removed from embryonic day 18 rats (Sprague Dawley), dispersed in a papain solution, triturated, and plated on polylysine-coated glass or plastic substrates. Details are found in our previous papers (Obrietan and van den Pol, 1996; van den Pol et al., 1996).

**Fura-2 digital imaging.** From 3 to 8 d after plating, cells on coverslips were loaded with fura-2 AM (Molecular Probes, Eugene, OR) and imaged on a Nikon inverted microscope with an Olympus 40× objective with high 340 nm light transmittance. A Sutter Lambda 10 filter wheel was controlled by Fluor software from Universal Imaging. A rapid perfusion chamber was used, allowing complete washout of agonists within a few seconds. Calcium standards (Molecular Probes) were used to calibrate the system according to the equation of Grynkiewicz et al. (1985). Additional details have been described previously (van den Pol et al., 1996, 1997).

**Whole-cell recording in cultured neurons.** Neurons were recorded with patch pipettes (4–6 M $\Omega$  size tip). An EPC7 amplifier was used with Axodata and IgorPro software. Two types of whole-cell recording were used. In conventional recordings, after obtaining a gigohm seal on the membrane, negative pressure was applied, and whole-cell recordings were obtained. For conventional whole-cell recording, the pipette solution contained (in mM): KMeSO<sub>4</sub> 116, KCl 27, MgCl<sub>2</sub> 1, HEPES 10, and EGTA 1.1, Mg-ATP 4, GTP 0.5, pH 7.3, with KOH. To avoid disturbing the normal intracellular Cl<sup>-</sup> concentration (Reichling et al., 1994; Ebihara et al., 1995) and to demonstrate the depolarizing actions of GABA, we also used gramicidin perforations for whole-cell recordings, as described in detail in previous papers (Chen et al., 1996; van den Pol et al., 1996; Gao et al., 1998). The pipette solution for gramicidin-perforated recording contained (in mM): KCl 145, MgCl<sub>2</sub> 1, HEPES 10, EGTA 1.1, and 50–100  $\mu$ g gramicidin, pH 7.3, with KOH. The recording chamber was continuously perfused at a rate of 1.5–2 ml/min with a bath solution containing (in mM): NaCl 155, KCl 2.5, CaCl<sub>2</sub> 2, HEPES 10, and glucose 10, pH 7.3, with NaOH.

**Brain slice whole-cell recording.** Hypothalamic slices from postnatal day 0 (P0)–P5 rats were prepared by conventional techniques. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and then decapitated. Their brains were rapidly removed and immersed in cold (1–3°C), oxygenated choline chloride solution (containing in mM: choline chloride 135, KCl 1, NaHCO<sub>3</sub> 20, NaH<sub>2</sub>PO<sub>4</sub> 1.2, dextrose 10, and MgSO<sub>4</sub> 20) for 1 min. Coronal slices (400  $\mu$ m) were cut with a vibratome, trimmed to contain just the hypothalamus, and then placed in a fluid–gas interface-type chamber humidified with 95% O<sub>2</sub> and 5% CO<sub>2</sub> and maintained with a constant flow of artificial CSF (ACSF) at 31–32°C. The ACSF contained (in mM): NaCl 124, KCl 3, CaCl<sub>2</sub> 2, NaHCO<sub>3</sub> 26, MgSO<sub>4</sub> 1.3, NaH<sub>2</sub>PO<sub>4</sub> 1.25, and glucose 11 equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, pH 7.2–7.4. Slices were allowed to recover for ~2 hr before recording.

Whole-cell recordings in slices were obtained using patch pipettes (4–7 M $\Omega$ ) pulled on a Flaming–Brown puller (Sutter Instruments) and filled with (in mM) KCl 145, MgCl<sub>2</sub> 1, HEPES 10, EGTA 1.1, Mg-ATP 4, and Na<sub>2</sub>-GTP 0.5. An Axoclamp-2A amplifier was used with Axodata and IgorPro software. In all experiments cells were kept at hyperpolarized membrane potentials of at least –75 mV (–75 to –95 mV) by applying a steady hyperpolarizing current. Cells were included in this study only if they had input resistances  $\geq$ 100 M $\Omega$  and had action potentials overshooting 0 mV. In experiments in which ionotropic glutamate receptors were blocked, CNQX (25  $\mu$ M) and AP5 (50  $\mu$ M) were applied to the bath. To apply bicuculline, L-AP4, and control vehicles, a Picospritzer was used with single- or double-barreled micropipettes. In experiments examining spontaneous depolarizing PSPs, a single drop of bicuculline (30  $\mu$ M) or L-AP4 (100  $\mu$ M) was applied to the surface of the slice. When examining evoked responses, a bipolar stimulating electrode made of teflon-coated platinum–iridium wire (75  $\mu$ m) was used to deliver electrical stimulations (50–500  $\mu$ A; 0.1–0.3 msec; 0.1 Hz). Cells were used for these experiments only if electrical stimulation could consistently evoke a PSP before the application of L-AP4.

L-CCG-I ((2S-1'S-2'S)-2-(carboxycyclopropyl)glycine from Tocris Cookson, St. Louis, MO) and L-AP4 (L-2-amino-4-phosphonobutyrate

from Research Biochemicals, Natick, MA) were used to activate the group I/II and III metabotropic glutamate receptors, respectively (Schoepp, 1994; Pin and Duvoisin, 1995; Roberts, 1995). Bicuculline, AP5, and CNQX were obtained from Research Biochemicals.

**Northern blots.** RNA from hypothalamus, hippocampus, olfactory bulb, cortex, cerebellum, and whole brain was purified as described elsewhere, and 10  $\mu$ g was loaded onto the gel (Ghosh et al., 1997). Northern hybridization was done individually with both DNA restriction fragments isolated from cDNA clones (kindly provided by Dr. S. Nakanishi) and PCR-amplified small DNA fragments synthesized by using each metabotropic glutamate receptor-specific primer on PCR-amplified cDNA templates isolated from rat whole-brain total RNA. The results were identical using both sets of probes. The Northern results presented here are the results using PCR-amplified small DNA fragments, because these probes gave cleaner results with very little background versus restriction enzyme digested larger DNA fragments. Details are found in our previous papers (van den Pol et al., 1994; Ghosh et al., 1997).

## RESULTS

Both calcium digital imaging with fura-2 and whole-cell patch clamp recording with conventional or gramicidin perforations were used to examine parallel aspects of glutamate inhibition in developing neurons in neonatal slices from the developing hypothalamus and from cultured hypothalamic neurons.

### High levels of spontaneous GABA activity in developing hypothalamic slices

In electrophysiological experiments using hypothalamic slices, KCl patch electrodes were used to determine whether spontaneous GABA-mediated events were present in the neonatal (P0–P5) mediobasal hypothalamus, focusing on arcuate–ventromedial nucleus neurons. The mean resting membrane potential of these developing neurons ( $n = 9$ ) was  $-54 \pm 4.8$  (SD) mV; to increase the ionic driving force, cells were sometimes held between –70 and –90 mV, which facilitated detection of PSPs. Input resistance in these cells was  $586 \pm 250$  (SD) M $\Omega$ . In 11 of 11 neurons recorded in normal ACSF, large depolarizing events were observed. These PSPs ranged in frequency from ~1 to 5 Hz and were reversibly blocked by application of the GABA<sub>A</sub> receptor antagonist bicuculline (30  $\mu$ M;  $n = 5$  of 5 neurons examined; Fig. 1), suggesting that they were GABAergic in nature. To determine whether these events were dependent on ionotropic glutamate receptor activation, additional experiments were performed in the presence of the kainate–AMPA and NMDA receptor antagonists CNQX (25  $\mu$ M) and AP5 (50  $\mu$ M), respectively. In this condition, large depolarizing PSPs (frequency 0.4–4 Hz) were still observed in 12 of 13 neurons examined and were blocked by bicuculline (30  $\mu$ M;  $n = 3$ ; Fig. 1). Unlike the adult arcuate nucleus region, in which glutamate provides an important driving force for GABA activity (Belousov and van den Pol, 1997), in the developing hypothalamus, GABA-mediated potentials are not dependent on classical fast glutamatergic neurotransmission but instead occur spontaneously and frequently.

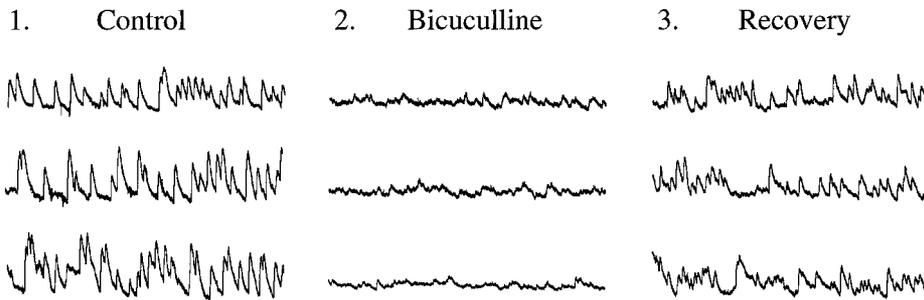
### GABA-evoked calcium rises are depressed by glutamate

#### Calcium imaging

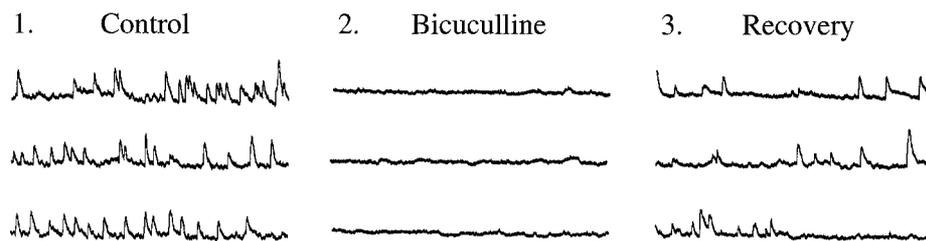
GABA and glutamate (each 5  $\mu$ M) evoke a Ca<sup>2+</sup> rise in developing neurons in studies with fura-2 digital imaging. In approximately one-third of the cells tested at 3–4 days *in vitro* (DIV), GABA evoked a greater Ca<sup>2+</sup> rise than glutamate did (33% of 119 neurons). In most of these neurons (85% of 39 neurons), the addition of glutamate to the GABA-containing solution depressed the Ca<sup>2+</sup> rise evoked by GABA (Fig. 2), suggesting that glutamate inhibited the effectiveness of GABA in generating Ca<sup>2+</sup> rises.

## Spontaneous GABA-mediated PSPs in neonatal hypothalamic slices

## A ACSF



## B. ACSF with AP5 and CNQX



**Figure 1.** Spontaneous GABA-mediated postsynaptic potentials in neonatal hypothalamic slices. *A*, Spontaneous depolarizing PSPs detected with whole-cell recording in a P1 arcuate-ventromedial nucleus (ARC-VMH) neuron in normal ACSF were reversibly blocked by the addition of the GABA<sub>A</sub> receptor antagonist bicuculline (30 μM). After bicuculline washout, PSPs recover. *B*, Large depolarizing PSPs were also observed in neonatal ARC-VMH neurons in the presence of AP5 (50 μM) and CNQX (25 μM) and were reversibly blocked by the addition of bicuculline (30 μM). This observation suggests that in the developing hypothalamus GABA-mediated activity is not dependent on ionotropic glutamate receptor activation. The traces in *B* were obtained in a hypothalamic slice from a P2 rat. Recordings in *A* and *B* were performed using KCl electrodes, and the membrane potential of these neurons was held at ~90 mV throughout the respective experiments.

Immunostaining for metabotropic glutamate receptors has shown widespread expression in different regions of the adult and developing hypothalamus (van den Pol, 1994; van den Pol et al., 1994, 1995). To test the hypothesis that the glutamate-mediated reduction in GABA-evoked Ca<sup>2+</sup> rise was in part caused by activation of metabotropic glutamate receptors (mGluRs), we used two group I/II nonselective mGluR agonists, *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylate (*t*-ACPD; Research Biochemicals), and (2*S*,1'*S*,2'*S*)-2-carboxycyclopropyl-glycine (L-CCG-I; Tocris Cookson). The Ca<sup>2+</sup> rise in response to GABA plus the mGluR agonist was compared with the Ca<sup>2+</sup> rise in response to GABA (5 μM) alone. Using as a criterion a decrease in the Ca<sup>2+</sup> elevation by at least 20%, we found that CCG evoked a substantial inhibition of the GABA-mediated Ca<sup>2+</sup> rise (Fig. 3). Twenty-three percent of 104 neurons tested showed a CCG-mediated reduction in GABA-evoked Ca<sup>2+</sup>. Because not all cells may express CCG-sensitive mGluR receptors, we compared the 20% of the cells showing the greatest inhibition in each group (CCG-treated or a second application of GABA as a control). The group (*n* = 21) that received GABA plus CCG showed a 34% (±2% SEM) reduction of its response to GABA alone. This difference was highly significant (*t* test; *p* < 0.0001). *t*-ACPD generated a modest inhibition (not statistically significant) of GABA-evoked Ca<sup>2+</sup>, with 14% of 103 cells showing a response decrement >20%. As a control for the reproducibility of the GABA-mediated Ca<sup>2+</sup> rise, we added GABA a second time. Only 6% of the cells (*n* = 104) showed a change >20%. When compared with a group (*n* = 32) receiving GABA plus kainate (5 μM), the group treated with GABA plus CCG showed a much greater decrease (*t* test; *p* < 0.0001). Thus, activation of a CCG-sensitive mGluR inhibited GABA-mediated Ca<sup>2+</sup> rises, probably at a postsynaptic site, as described for mGluR activation in other systems (Lachica et al., 1995).

## Presynaptic inhibition of GABA release by group III metabotropic glutamate receptor

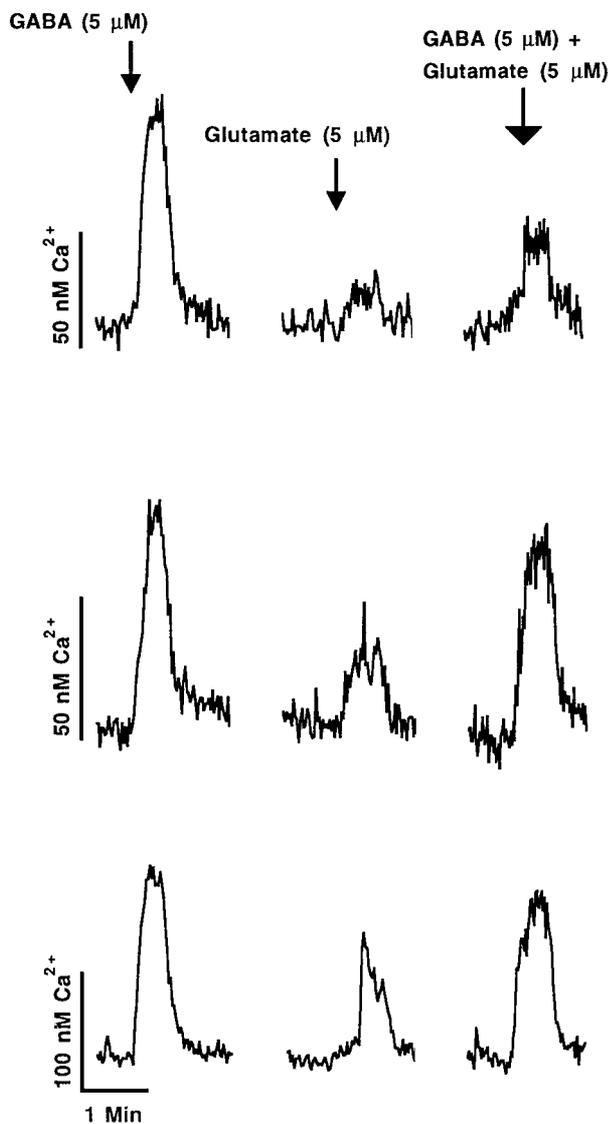
## Calcium imaging

Using fura-2 digital imaging we analyzed the influence of a group III mGluR agonist, L-AP4 (100 μM; Research Biochemicals), on bath-applied GABA-evoked Ca<sup>2+</sup> rises. Muscimol (5 μM), a GABA<sub>A</sub> agonist, evoked a Ca<sup>2+</sup> peak of 130 ± 9 nM (SEM); in the presence of L-AP4 the Ca<sup>2+</sup> rise was not altered by a substantial amount (117 ± 9 nM (SEM); *n* = 46; statistically insignificant change), indicating little postsynaptic inhibition by L-AP4 (see Fig. 4*B*). L-AP4 also had no direct effect on Ca<sup>2+</sup>; in the presence of tetrodotoxin (TTX, 1 μM) to block action potential-mediated transmitter release, the Ca<sup>2+</sup> levels were the same in the presence of L-AP4 (82 ± 2 nM) as in the absence of L-AP4 (84 ± 2 nM, SEM) (*n* = 125), suggesting that L-AP4 had no independent effect on calcium regulation in these neurons (Fig. 4*A*).

To examine the action of L-AP4 on synaptic release of GABA in the absence of TTX, all experiments were done in the presence of D,L-AP5 (100 μM) and CNQX (10 μM) to block glutamate receptor activation. In this situation, Ca<sup>2+</sup> transients were caused by synaptic release of GABA; bicuculline (30 μM) blocked the transients (Fig. 4*C,D*). Before addition of L-AP4 the mean Ca<sup>2+</sup> rise after bicuculline withdrawal was 98 ± 7 (SEM) nM. When L-AP4 was bath-applied, the Ca<sup>2+</sup> level decreased to 73 ± 6 (SEM) nM, a statistically significant decrease (*p* < 0.01; *n* = 65). Fifty-one percent of 65 neurons showed a depression (>20%) of the GABA-evoked Ca<sup>2+</sup> rise in the presence of L-AP4 (Fig. 4*C-E*).

## Inhibitory action of L-AP4 in developing spinal cord and cortical neurons

The above experiments were based entirely on hypothalamic neurons. To test the hypothesis that activation of group III mGluRs would depress excitatory GABA actions in neurons from other

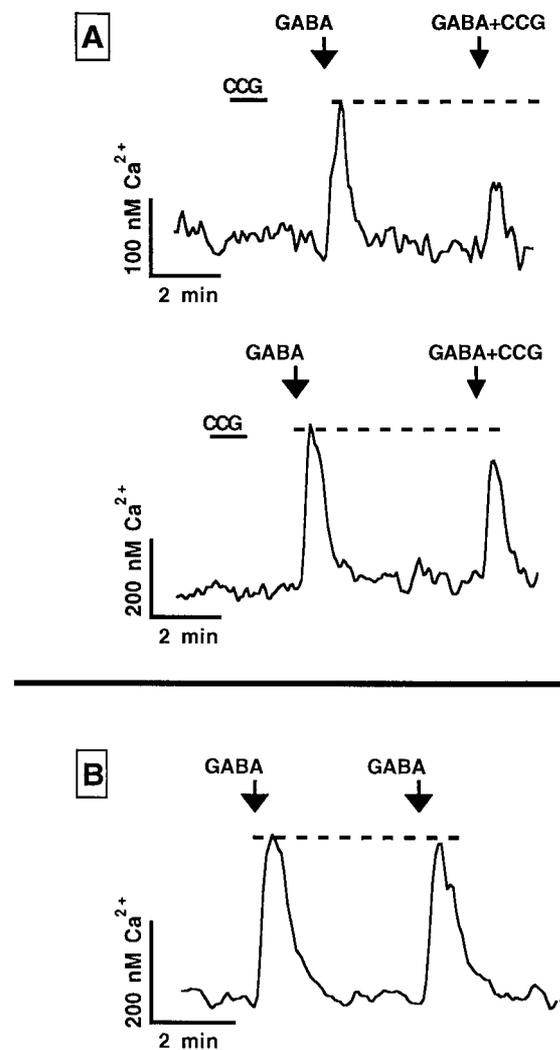


**Figure 2.** Glutamate reduces the amplitude of GABA-mediated calcium rises. In three hypothalamic neurons recorded simultaneously with digital fura-2 imaging after 3 d *in vitro*, both GABA (5  $\mu$ M) and glutamate (5  $\mu$ M) evoked  $Ca^{2+}$  rises. In these neurons the rise evoked by GABA is of greater amplitude than that evoked by glutamate. The addition of glutamate to GABA reduced the amplitude of the GABA-evoked  $Ca^{2+}$  rise. Each transmitter was applied for 30 sec and allowed a 5 min recovery time before the next transmitter application.

parts of the CNS, we also examined 4–6 DIV cultures of cortex or spinal cord neurons. In both regions we found an inhibition of the  $Ca^{2+}$ -elevating actions of GABA by L-AP4 (50  $\mu$ M). Based on cells that showed at least a 20 nM rise in  $Ca^{2+}$  in response to bicuculline washout, 13 of 21 spinal cord neurons and three of four cortical neurons showed a decrease in  $Ca^{2+}$  in the presence of L-AP4 (Fig. 5). All experiments were done in the presence of ionotropic glutamate receptor antagonists. In the presence of bicuculline (20  $\mu$ M), no effect of L-AP4 was detected, suggesting that the L-AP4 actions were dependent on GABA acting at a  $GABA_A$  receptor.

#### Metabotropic glutamate receptor inhibition of GABA activity in slices of developing hypothalamus

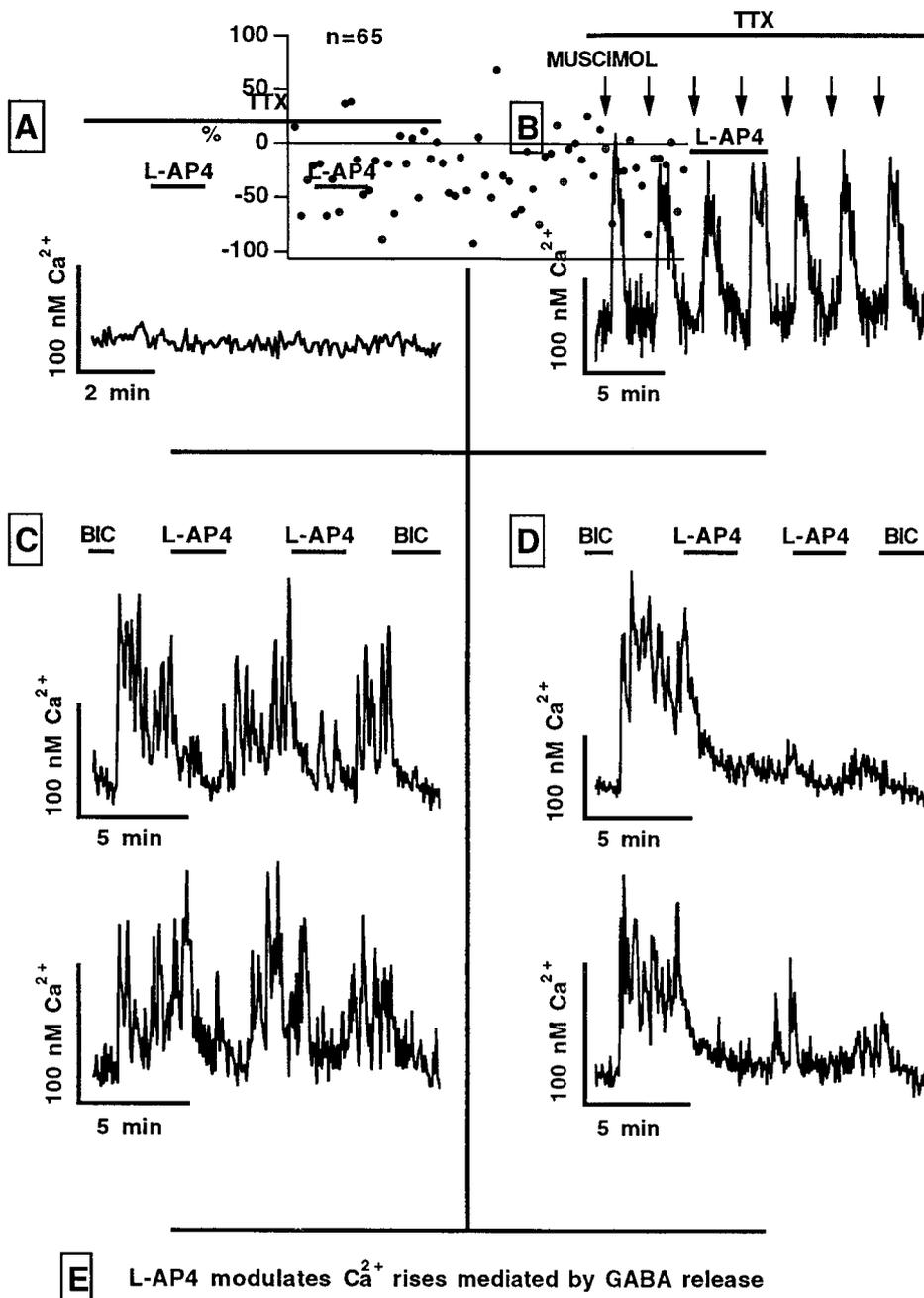
To test the hypothesis that metabotropic glutamate receptor activation suppresses  $GABA_A$ -mediated activity in neonatal hypothalamic



**Figure 3.** Group II metabotropic glutamate receptor agonist reduces  $Ca^{2+}$  response to GABA. *A*, Two neurons from different hypothalamic cultures show no direct  $Ca^{2+}$  response to the group II metabotropic glutamate receptor agonist CCG alone, but when CCG (50  $\mu$ M) is added together with GABA (5  $\mu$ M), a substantial decrease in the GABA-evoked  $Ca^{2+}$  rise is found. *B*, In a control experiment, a typical cell showed similar amplitude  $Ca^{2+}$  rises in response to GABA (5  $\mu$ M).

amic slices, we examined the effect of micropipette application to the slice surface (Christian and Dudek, 1988) of the selective mGluR agonist L-AP4 (100  $\mu$ M) on the frequency of spontaneous  $GABA_A$ ergic PSPs in ACSF containing CNQX (25  $\mu$ M) and AP5 (50  $\mu$ M). Using as a criterion PSPs  $\geq 10$  mV in amplitude, we found that L-AP4 caused a reversible suppression of the frequency of spontaneous  $GABA_A$ ergic PSPs in all arcuate and ventromedial nucleus neurons examined ( $n = 7$ ) by at least 40% (range, 40–90% reduction; mean decrease,  $60 \pm 6.9\%$  SEM) (Fig. 6*A*). In contrast, application of the control vehicle ( $n = 2$ ) had little effect, thus demonstrating the specificity of L-AP4 actions.

To further test this hypothesis, we also examined the effect of L-AP4 on evoked responses in ACSF containing CNQX and AP5. In five of five arcuate nucleus cells recorded, L-AP4 reversibly caused either a complete blockade or a substantial reduction of the amplitude of PSPs generated by electrical stimulation in an area at the border of the arcuate and ventromedial nuclei (Fig. 6*B*, *C1–C3*). At a baseline membrane potential of  $-70$  mV, L-AP4



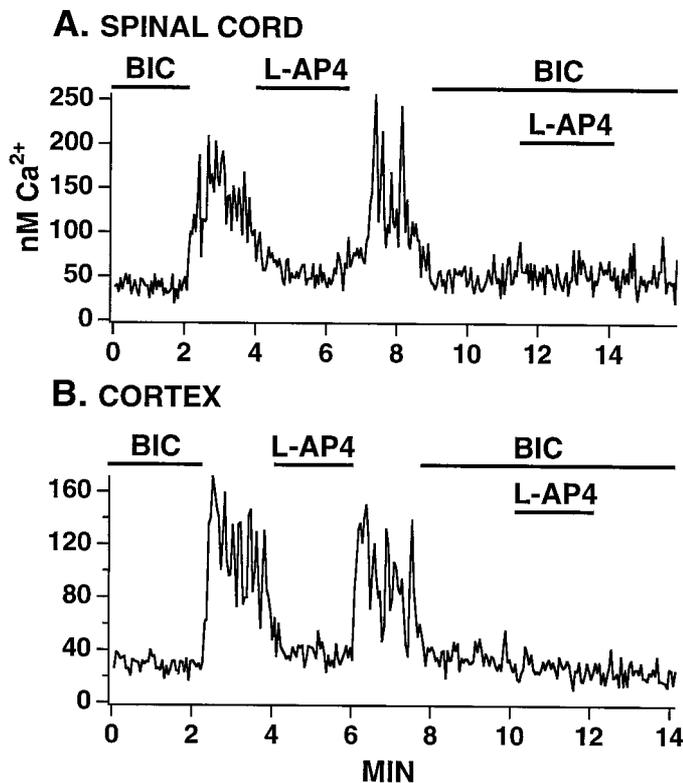
**Figure 4.** Group III metabotropic receptor activation modulates GABA-elevating actions presynaptically but not postsynaptically. *A*, L-AP4 (100  $\mu M$ ), a group III metabotropic glutamate receptor agonist, elicited no  $Ca^{2+}$  change in the presence of tetrodotoxin (TTX; 1  $\mu M$ ) in cultured hypothalamic neurons. *B*, L-AP4 (100  $\mu M$ ) did not modulate  $Ca^{2+}$  rises in response to the GABA<sub>A</sub> receptor agonist muscimol (5  $\mu M$ ), depicted in a typical neuron that showed a  $Ca^{2+}$  rise in response to muscimol in the presence of TTX (1  $\mu M$ ). These data suggest L-AP4 has little detectable postsynaptic effect on modulating GABA actions. *C*, In the presence of glutamate receptor antagonists AP5 (100  $\mu M$ ) and CNQX (10  $\mu M$ ), synaptically released GABA-generated  $Ca^{2+}$  rises were blocked by the GABA<sub>A</sub> receptor antagonist bicuculline (BIC; 30  $\mu M$ ). L-AP4 reduced the  $Ca^{2+}$  activity in both cells recorded at the same time. Because we found no postsynaptic modulation of GABA by L-AP4, this suggests an inhibition of GABA release from presynaptic axonal terminals. *D*, Some neurons showed an extended L-AP4 depression of GABA release as evidenced by the relative lack of recovery after L-AP4 washout. *E*, This scatterplot shows the effect of L-AP4 on  $Ca^{2+}$  raised by synaptically released GABA. The values were determined by the ratio  $Ca^{2+}$  in control (pre-L-AP4) to the  $Ca^{2+}$  value during L-AP4, and are represented by the percent change. Values below zero show the percent inhibition of L-AP4 on  $Ca^{2+}$  levels mediated by synaptically released GABA. Each point represents the percent change in  $Ca^{2+}$  for a single neuron. The zero (0) baseline represents the pre-L-AP4 level for a particular cell.

reduced the amplitude of the evoked GABA-mediated PSP by  $90 \pm 4.1\%$  (SEM). No significant change was observed in input resistance during L-AP4 application ( $p > 0.05$ ;  $t$  test), suggesting that the recording remained stable and that L-AP4 does not have a direct postsynaptic effect on ion channels on the cell body; this does not preclude the possibility that L-AP4 may modulate the

opening of somatodendritic ion channels in response to other factors not studied here.

#### Electrophysiology of cultured neurons

In electrophysiological experiments parallel to those described above with  $Ca^{2+}$  digital imaging, we examined the effect of L-AP4



**Figure 5.** Group III metabotropic receptor agonist depresses excitatory GABA actions in cortex and spinal cord neurons. In spinal cord (*A*) and cortical (*B*) neurons cultured for 4–6 d, and in the presence of AP5 (100  $\mu$ M) and CNQX (10  $\mu$ M), bicuculline (20  $\mu$ M) depressed calcium levels, indicating a dependence on synaptic GABA activity. L-AP4 (50  $\mu$ M) caused a strong decrease in calcium levels that recovered after L-AP4 washout. In the presence of bicuculline (30  $\mu$ M), L-AP4 had no effect on cytosolic calcium, suggesting that its effect was dependent on the excitatory actions of GABA.

(100  $\mu$ M) on spontaneous GABA-mediated postsynaptic currents. These experiments were done in the presence of D,L-AP5 (100  $\mu$ M) and CNQX (10  $\mu$ M) to block glutamate actions. In voltage clamp ( $-60$  mV holding potential), L-AP4 caused a substantial reduction in the frequency of spontaneous GABA-mediated EPSCs in five of six neurons, with a mean reduction by  $30 \pm 4\%$  (mean  $\pm$  SEM) (Fig. 7). L-AP4 evoked a maximum inhibition of 41% and a minimum inhibition of 20%. This decrease in EPSC frequency was statistically significant (*t* test;  $p < 0.01$ ). Consistent with the  $Ca^{2+}$  imaging and slice electrophysiology data, no independent effect of L-AP4 (100  $\mu$ M) on membrane potential was found. No difference in the postsynaptic current evoked by GABA (5  $\mu$ M) was found in the presence or absence of L-AP4 (100  $\mu$ M) ( $n = 4$ ) (data not shown), indicating a lack of effect of L-AP4 on postsynaptic actions of GABA and suggesting presynaptic actions, as addressed below.

#### Gramicidin recordings

Neurons in these experiments were recorded during early development, after 3–5 d *in vitro*, and at the period when GABA has depolarizing actions, in contrast to the hyperpolarizing actions found in mature neurons. Although GABA evoked inward currents in the experiments above, this in large part was caused by the choice of pipette solutions. We therefore did additional experiments to demonstrate that the inward postsynaptic currents would be found not only with conven-

tional whole-cell recording, but also in neurons with undisturbed intracellular  $Cl^-$  levels recorded with gramicidin-perforated patches (Myers and Haydon, 1972; Reichling et al., 1994; Ebihara et al., 1995). Gramicidin was added to the pipette solution, and whole-cell recordings were obtained, as previously described (van den Pol et al., 1997; Gao et al., 1998). Using gramicidin-perforated patches, neurons (70 of 70 cells in the present study and a previous paper by Gao et al., 1998) at this stage of development between 3 and 5 d *in vitro* show very consistent depolarizing responses to bath-applied GABA (5–30  $\mu$ M) and to synaptically released GABA. The mean resting membrane potential with gramicidin recordings was  $-50.9 \pm 12.3$  (SD) mV ( $n = 32$ ). In three of three neurons tested, L-AP4 (100  $\mu$ M) caused a  $32 \pm 3\%$  (mean  $\pm$  SEM) decrease in the frequency of GABA-mediated excitatory (inward) postsynaptic currents (data not shown). The frequency recovered after washout of the L-AP4. Parallel experiments with gramicidin-based recording have shown depolarizing actions of GABA in slices of developing cortex (Owens et al., 1996). Bicarbonate and  $Cl^-$  both pass through the GABA-gated anion channel (Kaila, 1994) and, in dendrites of mature dentate granule cells, the depolarizing actions of GABA are dependent on both  $Cl^-$  and bicarbonate (Staley et al., 1995). In contrast, as the depolarizing action is seen clearly in the present study in HEPES buffer lacking bicarbonate, only  $Cl^-$  appears to be necessary for the depolarizing actions of GABA in developing neurons (Obrietan and van den Pol, 1996); this does not argue against additional actions of bicarbonate but suggests they are not critical for the depolarizing action of GABA.

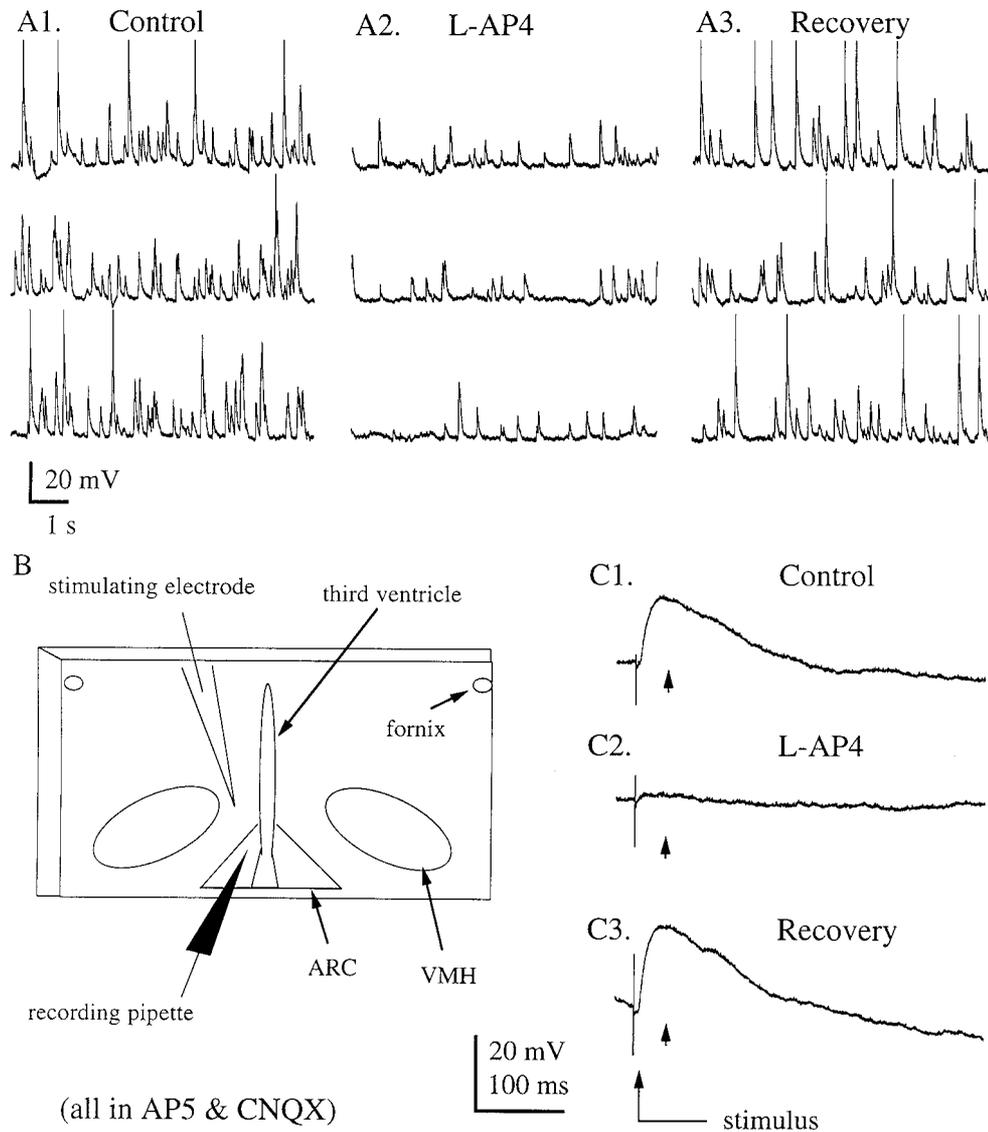
#### Miniature GABA-mediated EPSCs

To further demonstrate that the actions of L-AP4 were presynaptic, we used 1  $\mu$ M TTX to block action potentials, and recorded miniature EPSCs (mEPSCs) in the presence of AP5 (100  $\mu$ M) and CNQX (10  $\mu$ M) to block ionotropic glutamate receptor actions. In buffer that contained TTX, small mEPSCs, ranging in amplitude up to 40 pA were detected. These were blocked by bicuculline (30  $\mu$ M), showing that they were caused by GABA release from presynaptic axons. L-AP4 (100  $\mu$ M) caused a reversible reduction in the frequency of miniature GABA-mediated EPSCs in eight of eleven cells tested (Fig. 7), decreasing the mean GABA-mediated mEPSC frequency by  $51 \pm 5\%$  (mean  $\pm$  SEM) of pre-L-AP4 control levels, with a maximum decrease of 71% and a minimum decrease of 34% from control levels.

#### Ongoing inhibition of GABA excitation at group III metabotropic glutamate receptors

To test the hypothesis that there is an ongoing and spontaneous activation of group III mGluRs in hypothalamic neurons, we used a group III antagonist, *R,S*- $\alpha$ -methylserine phosphate (MSOP; 200  $\mu$ M), as previously described (Thomas et al., 1996; Faden et al., 1997; O'Leary et al., 1997). All experiments were done in the presence of AP5 (100  $\mu$ M) and CNQX (10  $\mu$ M) to block ionotropic glutamate receptors. Using voltage clamp recording, we first showed that in the presence of AP5 and CNQX, all inward currents could be blocked with bicuculline (30  $\mu$ M), indicating that they were caused by synaptic GABA release (Fig. 8*A*). When MSOP was added by bath application, we found that in six of six neurons tested, there was an increase in GABA activity (paired *t* test;  $p < 0.05$ ), with a mean increase in the frequency of PSCs of  $22 \pm 8\%$  and a maximum increase of 58% (Fig. 8*B*). After MSOP washout, the frequency of PSCs dropped to

## Metabotropic glutamate receptor activation suppresses spontaneous and evoked GABA-mediated PSPs in neonatal hypothalamic slices



**Figure 6.** Metabotropic glutamate receptor activation suppresses spontaneous and evoked GABA-mediated PSPs in the neonatal hypothalamic slice. **A**, The frequency of spontaneous GABAergic PSPs recorded from a postnatal day 4 ARC-VMH neuron was reversibly suppressed by the addition of L-AP4 (100  $\mu$ M). The membrane potential of this neuron was held at approximately  $-95$  mV throughout the experiment, and the spikes in **A1** and **A3** are clipped. **B**, Schematic diagram demonstrating the approximate configuration of stimulating and recording pipettes used in the experiments examining the effect of metabotropic glutamate receptor activation on evoked GABA-mediated PSPs. **C**, Application of L-AP4 (100  $\mu$ M) reversibly suppressed monosynaptically evoked GABA-mediated PSPs in a P5 ARC-VMH neuron. In this experiment monosynaptic PSPs (small arrow) were examined by delivering an electrical stimulus at the junction of the ventromedial and arcuate nuclei; L-AP4 showed a substantial reduction in PSP amplitude. The baseline membrane potential of this neuron was held at approximately  $-70$  mV in the examples shown here.

slightly below baseline levels (Fig. 8C). These data suggest that there is an ongoing activation of the mGluRs that inhibits GABA activity, and that when this is blocked an increase in GABA activity ensues.

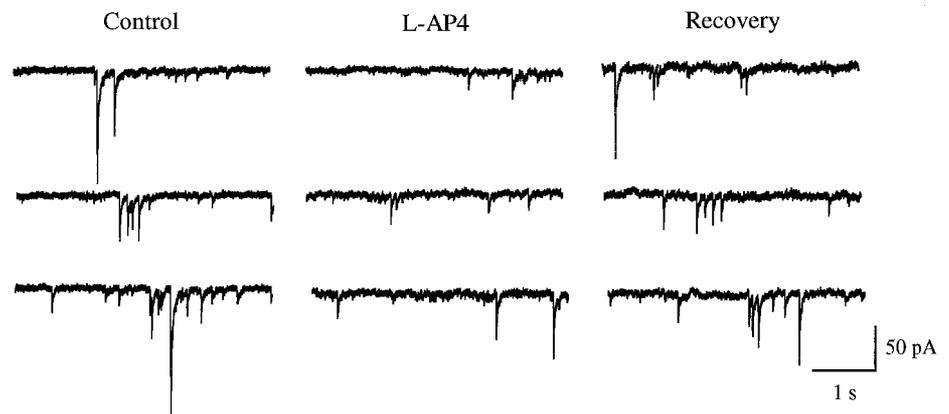
### Gene expression of metabotropic glutamate receptors in developing brain

Because the physiological experiments suggest an inhibitory role for metabotropic glutamate receptors, we prepared Northern blots of mRNA to study gene expression of the eight known metabotropic glutamate receptors (Nakanishi, 1994; Schoepp, 1994; Pin and Duvoisin, 1995) in a development series ranging from E15 embryos to adult (Fig. 9). Because each of the primary three groups of mGluRs is composed of two or more types, the Northern blots allow an analysis of which of the mGluRs within each group are expressed at a developmentally early time. These may be the ones underlying the early actions of group-specific

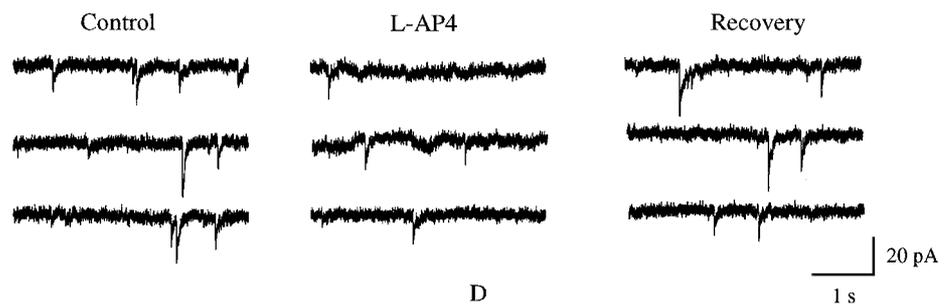
agonists found in the physiological experiments reported above with whole-cell recording and calcium digital imaging. A developmental series of hypothalamus was compared with hippocampus, cortex, olfactory bulb, cerebellum, and whole brain. Three different developmental Northern blots were made, and each showed similar results. The data from one of the blots are shown here, probed with each of the mGluRs, stripped, and then re-probed for the next. In parallel with the electrophysiology and calcium imaging results, analysis of the Northern blot (Fig. 9) suggests that many of the mGluRs were expressed very early in development. Even in embryonic brain tissue, expression of mGluR1, mGluR3, mGluR5, mGluR7a, and mGluR8 could be detected. By the day of birth (P1) expression of all three groups of mGluRs was found. At P1, group I (mGluR1 and mGluR5), group II (mGluR3), and group III (mGluR7a, mGluR7b, and mGluR8) are clearly seen in the hypothalamic lane and in many other brain regions.

## Group III mGluR activation reduces GABA-mediated PSCs presynaptically

## A GABA-mediated spontaneous postsynaptic currents (in CNQX and AP5)

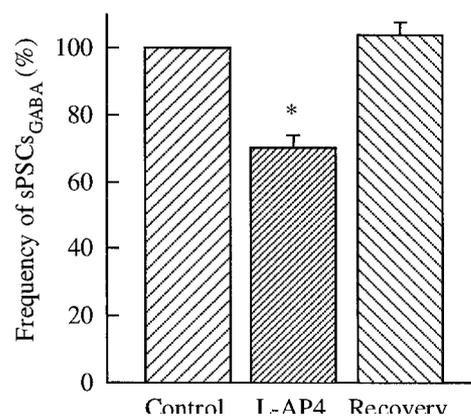


## B GABA-mediated miniature postsynaptic currents (in CNQX, AP5 and TTX)

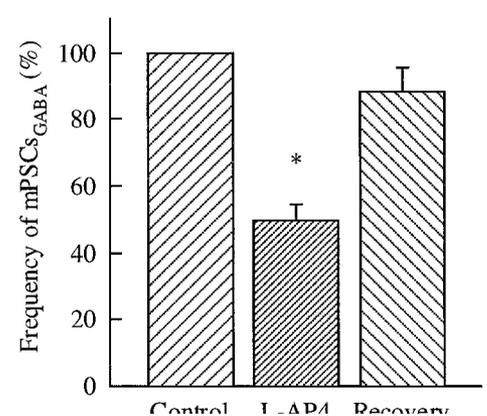


**Figure 7.** Group III metabotropic glutamate receptor activation reduces the frequency of spontaneous and miniature GABA-mediated EPSCs. *A*, In this typical hypothalamic neuron in culture, the addition of L-AP4 (100  $\mu$ M) reduced the frequency of spontaneous EPSCs in the presence of glutamate receptor antagonists AP5 (100  $\mu$ M) and CNQX (10  $\mu$ M). After L-AP4 washout, the frequency of EPSCs increased again (*Recovery*). *B*, An example of mEPSCs in the presence of AP5 (100  $\mu$ M), CNQX (10  $\mu$ M), and TTX (1  $\mu$ M). The frequency of mEPSCs is reduced by L-AP4. L-AP4 caused a statistically significant (*t* test;  $p < 0.05$ ) decrease in GABA-mediated EPSC frequency. *D*, The bar graph shows the mean frequency shift in GABA-mediated mEPSC frequency before, during, and after L-AP4 administration. The star denotes a statistically significant (*t* test;  $p < 0.05$ ) inhibition of mEPSC frequency in the presence of L-AP4 in 8 of 11 neurons.

C



D



## DISCUSSION

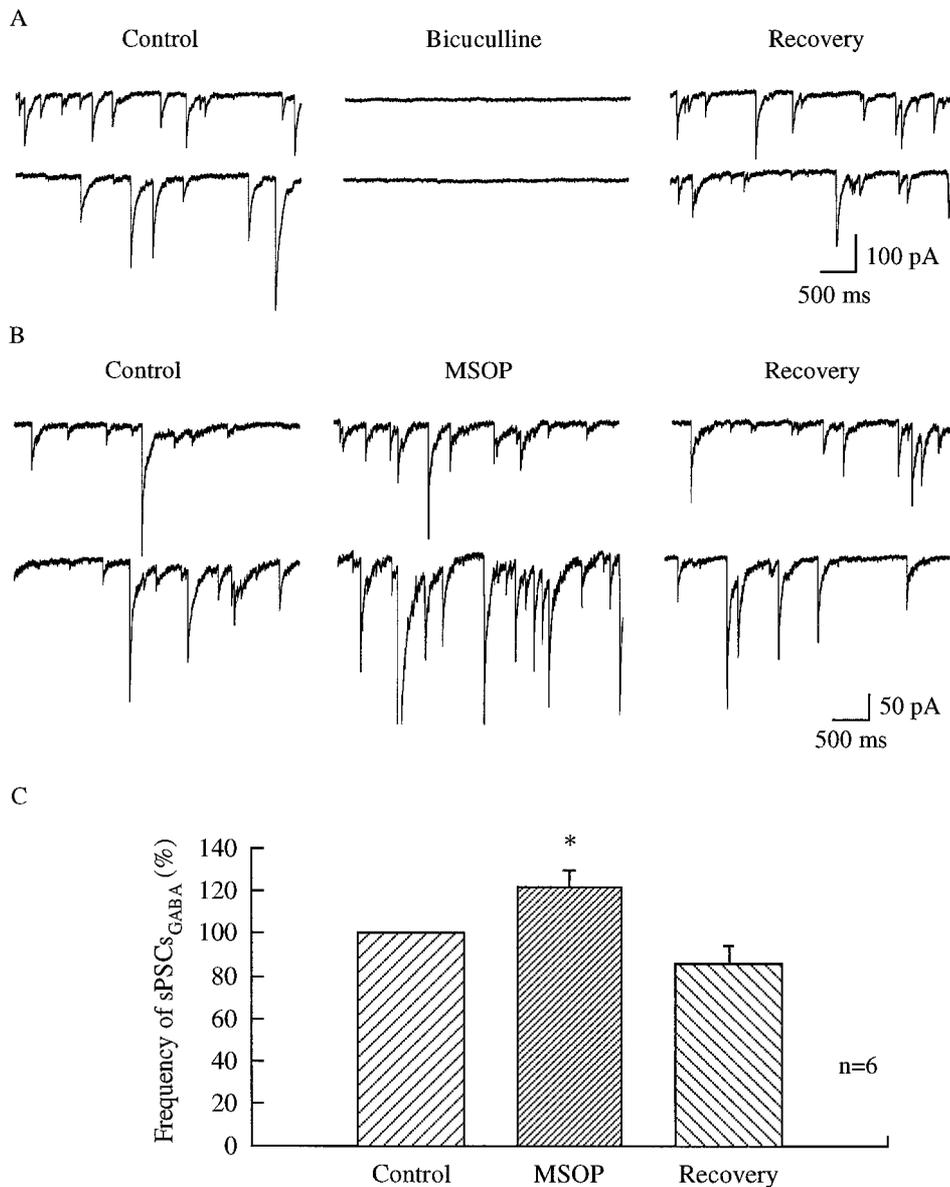
The data presented here suggest that during the developmental period when GABA exerts depolarizing actions, glutamate can and does act to reduce this excitatory activity at both presynaptic and postsynaptic sites. Postsynaptically, acting at a group I/II metabotropic receptor, glutamate inhibits the calcium increase in response to GABA-mediated depolarization. Presynaptically, acting at a group III metabotropic receptor, glutamate can reduce GABA release. Blocking the group III receptor leads to an increase in GABA activity, suggesting an ongoing inhibition of GABA activity by this glutamate receptor.

## 'Calcium regulation in developing neurons

What are the functional ramifications of a glutamate-mediated inhibition of GABA-regulated calcium rises? Regulation of cy-

tosolic calcium levels may be a critical aspect of the depolarizing actions of GABA, and these are not dependent on generating action potentials in a postsynaptic cell, but can be evoked simply by activation of voltage-dependent calcium channels. In fact, in a number of contexts, GABA activation of calcium influx could be of greater significance to developing neurons than evoking action potentials. Changes in cytoplasmic calcium can regulate gene expression (Vacarino et al., 1992), cell division of neuronal progenitor cells (LoTurco et al., 1995), migration of developing neurons (Komuro and Rakic, 1996), and programmed cell death (Lampe et al., 1995). Although no calcium-elevating effect of GABA was found in hippocampal neurites (Mattson and Kater, 1989), in developing hypothalamic neurons, GABA can act locally to increase calcium within restricted regions of neurites,

### Group III mGluR inhibition increases GABA-mediated PSCs (all in AP5 and CNQX)



**Figure 8.** Metabotropic glutamate receptor antagonist potentiates GABA activity. *A*, In the presence of AP5 (100  $\mu$ M) and CNQX (10  $\mu$ M), PSCs are completely blocked by bicuculline (30  $\mu$ M) in synaptically coupled hypothalamic neurons in culture. After washout, recovery is found. These data indicate that PSCs in these conditions are solely caused by synaptic release of GABA. In these experiments, KCl was used in the recording pipette. *B*, The group III antagonist MSOP (200  $\mu$ M) causes an increase in the frequency of GABA-mediated PSCs that recovers after MSOP washout. *C*, In this *bar graph*, the data from all six neurons are combined. Blocking of the group III mGluR by MSOP causes a significant ( $p < 0.05$ ; paired *t* test) increase in the frequency of GABA-mediated PSCs. After MSOP washout, the frequency of PSCs decreases. These data support the view that there is an ongoing inhibition of GABA activity in developing hypothalamic neurons by activated mGluR receptors.

growth cones, or developing neuronal perikarya (Obrietan and van den Pol, 1996). By increasing calcium, GABA can therefore alter neurite and growth cone extension and direction of growth (Mattson and Kater, 1987; Kater and Mills, 1991; Rehder and Kater, 1992; Zheng et al., 1994) and increase transmitter release. Local GABA-evoked calcium rises at specific regions of the plasma membrane may also play a role in GABA receptor protein aggregation, as shown for similar excitatory actions of glycine in developing spinal cord neurons (Kirsch and Betz, 1998). Because the specific level of cytosolic calcium may be critical for many of the actions described here, the ability of glutamate to reduce the amplitude of a calcium rise generated by GABA gives glutamate an important modulatory role.

GABA is found in high concentrations in growth cones of developing axons before synapse formation (van den Pol, 1997); if it is released from the growing axon, as the transmitter acetylcholine is released from neurites of developing motoneurons

(Hume et al., 1983; Young and Poo, 1983), then a growing GABAergic axon could potentially initiate communication with a prospective postsynaptic target before establishment of a synapse. It may be that the calcium-elevating actions of GABA constitute a critical part of the early stages of synapse formation. Based on data presented here, glutamate could theoretically inhibit this communication either by inhibition of GABA release from the developing axon or reduction of calcium influx into a potential postsynaptic partner for the growing GABAergic axon.

Glutamate inhibition of the excitatory actions of GABA would only occur during an early transient phase of neuronal development, but during this time period the inhibitory actions appear robust. In this paper we found that in almost all neurons that showed a greater calcium response to GABA than to glutamate, that together, glutamate would reduce the amplitude of the GABA-mediated calcium rise. We have previously shown that in very early stages of development, the majority of neurons show a

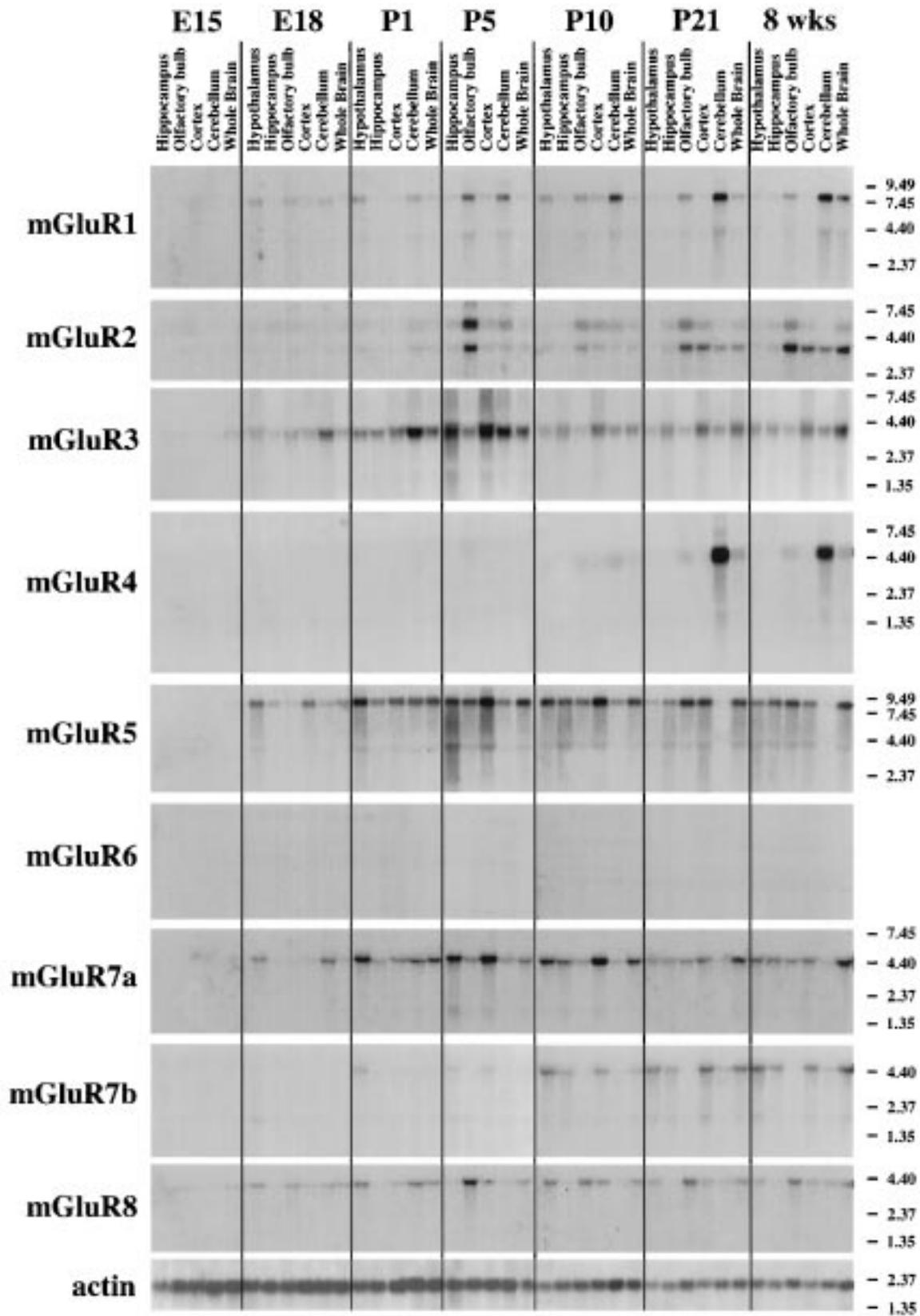


Figure 9. Metabotropic glutamate receptors: developmental expression examined with Northern blot. Ten micrograms of RNA samples were loaded from hippocampus, hypothalamus, cortex, cerebellum, olfactory bulb, and whole brain at different development time points from E15 to adult (8 weeks). Each lane is based in a mixture of tissue from three rats. Even in the embryonic brain at E18, expression can be detected for some of the mGluRs, suggesting early expression. At the bottom are control lanes showing actin RNA. The slightly higher level of expression of actin in developing brain can be interpreted as a greater level of actin synthesis rather than differential loading.

greater calcium rise in response to GABA than to equimolar concentrations of glutamate (Obrietan and van den Pol, 1995). This suggests that during the early period of hypothalamic development when the  $\text{Cl}^-$  reversal potential is positive to the membrane resting potential, the inhibition of GABA-mediated elevations in neuronal calcium by glutamate would be substantial.

Although under certain conditions the developing brain may be somewhat more excitable than the adult brain (Schwartzkroin, 1984; Hablitz, 1987; Swann et al., 1988), excessive activity is normally not found during development. One reason that the role of GABA as an excitatory transmitter in development does not result in runaway excitation when coupled with glutamate actions may be that glutamate can reduce GABA excitation, both at presynaptic and postsynaptic glutamate receptors. As neurons develop, the roles of GABA and glutamate change. Glutamate-evoked calcium rises increase in amplitude, whereas those evoked by GABA disappear as the  $\text{Cl}^-$  reversal potential gradually shifts in a negative direction (Obrietan and van den Pol, 1995; van den Pol et al., 1995; Chen et al., 1996).

### Metabotropic glutamate receptors

Our physiological data and Northern blot analysis showing that both presynaptic and postsynaptic metabotropic glutamate receptors are expressed and functionally active during early periods of development supports their potential role in the inhibitory modulation of early neuronal activity and synapse formation.

The physiological studies demonstrate that at least two groups of mGluRs may participate in inhibitory actions during development; the Northern blot analysis suggests that most of the mGluRs (Nakanishi, 1994), with the exception of mGluR6, are expressed during development and could contribute to the physiological actions found. Based on whole-cell recording in both cultured hypothalamic neurons and in slices of the developing hypothalamus, we find that activation of group III mGluRs causes a widespread inhibition of GABA action, primarily by presynaptic inhibition of GABA release. In fact, in hypothalamic slices, activation of group III mGluRs almost completely blocked (90% inhibition) the evoked GABA-mediated PSP in the arcuate nucleus region. Given the strong expression of mGluR7a mRNA we find in Northern blots of the developing hypothalamus, this may be the group III receptor involved in this inhibition. Together, these results are in strong support of the idea that mGluRs are expressed very early in neuronal development on presynaptic GABAergic axons that are in the process of establishing synapses. Thus, glutamate activation of these receptors would tend to reduce GABAergic excitation by inhibition of release. Expression of mGluR mRNA early in development is found not only in hypothalamus, but also to varying degrees in other brain regions examined for comparison, suggesting that many of the inhibitory actions of mGluRs on GABA-mediated excitation described in this paper relating to hypothalamic neurons may also be present in other brain areas, as supported by our parallel work on spinal cord and cortical neurons.

mGluR inhibition of release of GABA and other transmitters is not unique to developing neurons but has also been described in mature neurons in the hippocampus (Gereau and Conn, 1995), hypothalamus (Chen and van den Pol, 1998), and elsewhere (Nakanishi, 1994; Salt and Turner, 1996; Bonci et al., 1997; Schaffhauser et al., 1998). However, in mature neurons, inhibition of GABA would ultimately have a fundamentally different effect; reducing GABA-mediated inhibition would tend to increase excitation by disinhibition. A primary

point of our results is the fact that functional mGluRs are strongly expressed at a very early stage of neuronal development, during a period when synapse formation is just beginning. Furthermore, even at the earliest stages of synapse formation, mGluR activation can exert a powerful depressive action on excitatory GABA activity. Metabotropic glutamate receptors are found early in phylogeny, including in invertebrates in which they may act by similar mechanisms (Parnentier et al., 1996). Partial homology is even found with bacterial periplasmic binding proteins (O'Hara et al., 1993), indicating a possible early evolutionary origin. This raises the speculation whether early in evolution glutamate acting on mGluRs may have mediated inhibitory actions.

### Dual role for glutamate

In the present work we focus on inhibitory actions of glutamate on GABA excitation during the developmental maturation of hypothalamic neurons. These inhibitory actions of glutamate in developing neurons have not been addressed before. Under other conditions glutamate can also exert direct excitatory actions on developing hypothalamic neurons (Chen et al., 1995; van den Pol et al., 1995). In addition, during early development, glutamate, acting at AMPA-kainate receptors, can also act synergistically with GABA to evoke action potentials if glutamate receptor activation occurs after GABA-activated  $\text{Cl}^-$  channels have closed but the membrane potential is still partially depolarized (Gao et al., 1998). In the hippocampus, GABA can act to depolarize developing neurons, and by relieving the NMDA receptors of their voltage-dependent  $\text{Mg}^{2+}$  block, can thereby enhance glutamate-mediated depolarization (Ben Ari et al., 1994; Leinekugel et al., 1997). Together, these data show that glutamate can act either to inhibit or enhance the excitatory actions of GABA in developing neurons. During development, more synapses are produced than are needed; some are maintained, and others are lost. In line with a Hebbian model of synaptic strengthening, the dual role of glutamate (enhancing or reducing GABA excitation) may give it a crucial role in determining which GABAergic synapses get stabilized and which are lost. Because a principal role for GABA in the mature brain is to counteract glutamate-mediated excitation, it seems reasonable that glutamate may play some modulatory role in defining GABAergic synapse formation.

GABA can play a number of potentially important roles during development. These include regulation of neurite growth, synapse formation, growth cone guidance, cell division, and synapse stabilization. The ability of glutamate to inhibit the excitatory activity of GABA at both presynaptic and postsynaptic sites would allow glutamate to exercise an important modulatory role in early development. Later in development, glutamate would take on an increasing larger role as the primary excitatory amino acid transmitter.

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