GABA, Not Glutamate, a Primary Transmitter Driving Action Potentials in Developing Hypothalamic Neurons

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Gao, Xiao-Bing and Anthony N. van den Pol. GABA, not glutamate, a primary transmitter driving action potentials in developing hypothalamic neurons. J Neurophysiol 85: 425–434, 2001. Neuronal activity is critical for many aspects of brain development. It has often been assumed that the primary excitatory transmitter driving this activity is glutamate. In contrast, we report that during early development, synaptic release of GABA, the primary inhibitory neurotransmitter in the mature brain, is not only excitatory but in addition plays a more robust role than glutamate in generating spike activity in mouse hypothalamic neurons. Based on gramicidin perforated whole cell and extracellular recording, which leave intracellular Cl− unperturbed in brain slices and cultures, the GABA$_{A}$ receptor antagonist bicuculline induced a dramatic decrease in spike frequency (83% decrease) in developing neurons, three times greater than that generated by glutamate receptor antagonists 2-amino-5-phosphono-pentanoic acid and 6-cyano-7-nitroquinoxalene-2,3-dione. Thus a number of factors related to spike-dependent stabilization of neuronal connections, including Hebbian mechanisms, that are generally applied to glutamate transmission may also participate in stabilization of GABA circuits.

INTRODUCTION

Although synapses can develop in the absence of neuronal activity, a large number of reports have demonstrated the importance of neuronal activity, particularly action potentials, during brain development. Such activity is critical to define synaptogenesis and synaptic efficacy (Nakagami et al. 1997), regulate the expression of adhesion molecules (Itoh et al. 1997), define axon structure (Lin and Constantine-Paton 1998), prevent inappropriate connections (Jarecki and Keshishian 1995), allow the segregation of functionally related axons (Kalil 1990), facilitate development of inhibitory circuits (Seil and Drake-Baumann 1994), and, in general, facilitate the correct wiring of the brain and spinal cord (Kalil et al. 1986; Nelson et al. 1989). The absence of normal neuronal activity during development can lead to permanent dysfunction (Hubel and Wiesel 1998). Although transmitters may produce depolarizing activity at the cell body, only if spike threshold is reached does the neuron send the message down the axon to distant sites of synaptic termination; the action potential is critical for long distance signaling.

The assumption that has generally been held is that the excitatory transmitter glutamate is the principal mediator regulating neuronal activity and spike generation in the developing brain as it is in the mature brain. A number of interesting experiments have demonstrated an important role for glutamate in refining synaptic connections (Hofer and Constantine-Paton 1994; Lin and Constantine-Paton 1998). A large proportion of synapses in the brain use GABA (Gribkoff et al. 1999; Kim and Dudek 1992; Strecker et al. 1997; Tasker and Dudek 1993), and half of all presynaptic hypothalamic boutons contain immunoreactive GABA (Decavel and van den Pol 1990). GABA is the primary inhibitory transmitter in the mature brain. In the developing brain, GABA may exert depolarizing actions due to an elevated Cl− reversal potential as reported in many brain regions including hypothalamus, spinal cord, olfactory bulb, cerebellum, and hippocampus in both cultured neurons and brain slices (Ben-Ari et al. 1989; Chen et al. 1996; Leinekugel et al. 1997; LoTurco et al. 1995; Obata 1974; Owens et al. 1996; Reichling et al. 1994; Rohrbough and Spitzer 1996; Serafini et al. 1995; Wu et al. 1992). Furthermore during development, GABA has been found to enhance neurite outgrowth, increase synapse formation, alter cell division of neuronal precursors, modulate neuron migration, and influence growth cone dynamics, suggesting a developmental role for GABA neurotransmission (Barbin et al. 1993; Behar et al. 1996; LoTurco et al. 1995; Obrietan and van den Pol 1998).

The possibility that GABA-mediated depolarization under some circumstances leads to action potentials has been suggested and demonstrated (Andersen et al. 1980; Ben-Ari et al. 1989; Gao et al. 1998; Serafini et al. 1995). Although depolarizing events are often excitatory, they can also be inhibitory. Related to GABA, such activity has been called a conductance shunt type of inhibition, shunting inhibition, or depolarizing inhibition (Alger and Nicoll 1979; Andersen et al. 1980; Staley and Mody 1992). In some cells, GABA-mediated depolarization may be primarily inhibitory due to current shunt (Staley and Mody 1992) or may depress glutamate actions by transient shunting activity (Gao et al. 1998).

Based on previous work showing that in some circumstances GABA is capable of evoking action potentials, we tested the hypothesis that GABA is responsible for most spike activity and that, in contrast, the excitatory actions of glutamate relat-
ranging to spike generation are relatively weak during early development of hypothalamic neurons.

METHODS

Brain slices and cultures

Coronal hypothalamic slices, 400 μm thick, were cut on a vibratome from the developing brains of P1–P4 mice or from an older group of P8–P10 mice. Briefly, mice were anesthetized with pentobarbital sodium (Nembutal; 80 mg/kg) and then decapitated. The brains were rapidly removed and immersed in cold (4°C) oxygenated bath solution [containing (in mM): 150 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose, pH 7.3 with NaOH]. After being trimmed to contain only hypothalamus, the slices were transferred to a recording chamber. Slices were constantly perfused with bath solution at 2 ml/min. Rodent use was approved by the university committee on animal use.

To generate cultures, neurons were dissociated from E15 to E18 mouse embryonic hypothalami and spinal cords and cultured as described previously (Gao et al. 1998).

Immunocytochemistry

To detect GABA immunoreactive cells, 4–5 days after culturing neurons, some cultures (n = 6) were fixed with 3% glutaraldehyde and after membrane permeabilization with 0.3% Triton X-100 were immunostained with GABA antiserum made in rabbits. The specificity of the antiserum has been described previously (van den Pol 1997). After overnight incubation in the primary antiserum diluted 1:5,000, cultures were washed and then stained with a secondary antiserum of goat anti-rabbit immunoglobulin (Molecular Probes) conjugated to Texas Red and diluted 1:150. Cultures were viewed on an Olympus IX70 inverted microscope, and photomicrographs were taken with a Spot-2 digital camera interfaced with a lab computer. Contrast was adjusted in Photoshop, and micrographs were printed on an Epson 900 digital printer.

Extracellular recording

Extracellular recordings were made with a glass electrode (resistance = 1 MΩ) with a DAM 50 differential amplifier (World Precision Instruments) in the area of the arcuate and ventromedial nuclei in the mediobasal hypothalamus from P1–P4 and P10 mice. The band-pass was 10–3,000 Hz. All data were sampled at 500 Hz with an Apple Macintosh computer using AxoData 1.2.2 (Axon Instruments).

Whole cell patch-clamp recording

Experiments were performed at room temperature on cultured neurons after 4–7 days in vitro (DIV; young neurons) and after 20 DIV (mature neurons). Recordings were made from the area of the arcuate and ventromedial nuclei in hypothalamic slices. The recording chamber was continuously perfused at a rate of 1.5–2 ml/min with a bath solution containing (in mM): 150 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose, pH 7.3 with NaOH. The tip resistance of recording pipettes was 4–6 MΩ after being filled with pipette solution containing (in mM): 145 KC1, 1 MgCl2, 10 HEPES, 1.1 EGTA, 2 Mg-ATP, and 0.5 Na2-GTP, pH 7.3 with KOH. Gramicidin-perforated recording, which maintains a physiological Cl– concentration inside the recorded cell, was used in most experiments except where otherwise mentioned, as previously described (Gao et al. 1998). The series resistance after 20–30 min gramicidin perforation was between 40 and 50 MΩ. Experiments were performed under current or voltage clamp.

In some experiments GABA or glutamate was applied to recorded neurons either through a micropipette placed 2–4 μm away from the recorded cell or by application through a large flow pipe aimed at the recorded cell. All data were sampled at 3–10 kHz and filtered at 1 kHz with an Apple Macintosh computer using AxoData 1.2.2 (Axon Instruments). 2-Amino-5-phosphono-pentanoic acid (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and bicuculline (BIC) were obtained from RBI (Research Biochemical International).

All data are reported as means ± SE. Because of the variation in the frequency of action potentials or spikes among different recorded neurons, all data were normalized to the percentage of control frequency before further statistical analysis. ANOVA was used to determine levels of statistical significance when three groups of data were compared, together with a post hoc Scheffe comparison; Student’s t-tests were used for comparison of two groups of data.

RESULTS

GABA immunocytochemistry

To detect the presence of GABAergic neurons in our hypothalamic cultures, immunocytochemically labeled cells were examined. After 5 DIV, a time when many of our recordings described in the following text were done, GABA immunoreactive cells and processes were common. Long, thin immunoreactive axons extended from immunoreactive cells bodies and boutons made contact with both GABA immunoreactive (Fig. 1A) and nonimmunoreactive neurons. Astrocytes showed no GABA immunoreactivity, and a number of cells with the appearance of neurons also lacked GABA immunostaining. Cells and processes showing no GABA immunoreactivity were detected using green fluorescent protein as a marker for cells in general (Fig. 1B), as described previously (Gao and van den Pol 2000). Approximately 35–40% of the neurons showed GABA immunoreactivity after 5 DIV. Whether additional developing neurons in culture synthesized GABA, but at levels too low to be detected with immunostaining, remains to be determined. Control experiments with preabsorption of the antiserum with GABA conjugated to a protein carrier, or omission of the primary antiserum, resulted in an absence of immunolabeling. These results with mouse cultures are parallel to similar experiments done with GABA immunostaining of rat neurons (van den Pol 1997).

GABA, not glutamate, drives action potentials in developing hypothalamic neurons

Initial experiments were done with conventional whole cell recording in which the pipette solution contained 29 mM [Cl–], to mimic the intracellular chloride concentration previously reported in developing rat hypothalamic neurons of similar age (Chen et al. 1996). The resting membrane potential ranged from −31.6 to −57.5 mV with an average of −46.1 ± 2.0 mV (n = 12). During the application of the GABA A receptor antagonist bicuculline (30 μM; BIC), the frequency of action potentials decreased from 20 ± 6 to 2 ± 1/min (90% reduction) and returned to 15 ± 6/min after BIC washout.

As the responses to GABA are dictated to a large degree by intracellular Cl–, to leave intracellular Cl– undisturbed by the pipette solution, action potentials were recorded with gramicidin-perforated whole cell recording (Chen et al. 1996; Gao et al. 1998) under current clamp in 4–7 DIV hypothalamic neurons. Neurons had an average resting membrane potential of −44.6 ± 1.69 mV (range −38.5 to −52.8 mV, n = 9). Spike frequency was based on a 1-min sample at the end of receptor...
antagonist application or after washout. After a 10-min recording of the control baseline, BIC (30 μM) was applied through a flow pipe. The frequency of action potentials decreased from a control level of 13 ± 2 to 0.3 ± 0.2/min and returned to 18 ± 10/min after BIC washout, indicating a decrease of 97.5 ± 1.5% in the frequency of action potentials (n = 9) when GABA_A receptors were blocked (Fig. 2A). Combining the results of gramicidin recordings with the conventional whole cell recordings, as each gave similar results, BIC decreased the frequency of action potentials by 93.1 ± 2.0% (n = 21), a very significant and substantial decrease (P < 0.01).

Gramicidin-perforated patch recordings were also made during the application of glutamate receptor antagonists CNQX and AP5 to hypothalamic neurons. The frequency of action potentials was decreased from 17 ± 3 to 10 ± 3/min and recovered after antagonist washout to 15 ± 4/min (Fig. 2A). Statistical analysis suggested that the decrease in the frequency of action potentials (34.6 ± 16.15%, n = 15) was significant (P < 0.01). The difference between the decrease caused by GABA and glutamate receptor antagonists was also significant (P < 0.01, ANOVA test). Thus these data are consistent with the concept that both GABA and glutamate contribute to spike generation during this developmental time period but that GABA played a greater role in triggering action potentials than glutamate did.

In older hypothalamic neurons (>20 DIV), gramicidin-perforated recordings were also performed (Fig. 2B). The frequency of action potentials dramatically decreased from 41 ± 26 to 5 ± 3/min and returned to 49 ± 18/min in the presence of CNQX and AP5, as expected. This decrease in the frequency of action potentials (75.4 ± 15.6%, n = 5; the percentage used here and elsewhere is based on the mean of percentages from each neuron, not on the spike frequency absolute value) was significant (P < 0.05). In contrast, the frequency of action potentials was not depressed but enhanced by treatment with BIC in the same neurons. Mean frequency increased from 40 ± 23 to 56 ± 26/min in BIC and returned to 47 ± 26/min after removal of BIC (P < 0.05; ANOVA). The difference between changes in the frequency of action potentials induced by block of GABA or glutamate receptors was significant (P < 0.01). Thus our data demonstrate that in mature hypothalamic neurons (>20 DIV) action potentials were mediated by glutamate receptors and GABA played an inhibitory role in spike generation, as expected.

**GABA excitation is independent of glutamate receptors in hypothalamic neurons**

Our data suggested that the generation of action potentials was dependent on the activation of GABA receptors during development of hypothalamic neurons. There are two possible mechanisms that may contribute to this phenomenon. The first one may be due primarily to inward current as Cl⁻ exits the neurons after GABA stimulation, leading to the initiation of action potentials if the depolarization induced by GABA receptor activation reaches the threshold for action potentials (Gao et al. 1998). The second possibility is that the depolarization induced by GABA receptor activation removes the voltage-dependent Mg²⁺ blockade of NMDA receptors and leads to NMDA receptor-dependent action potentials, as reported in hippocampal neurons (Leinekugel et al. 1997). In this scenario, GABA acts synergistically with glutamate to evoke spikes. Since GABA and glutamate receptor antagonists together block all synaptic currents in hypothalamic cultures and slices from P1 mice, the direct synaptic contribution of other neurotransmitters in the absence of GABA and glutamate is probably negligible. We tested the effect of the NMDA receptor antagonist AP5 on the frequency of action potentials in 4–7 DIV hypothalamic neurons.
After 10 min recording of baseline spike activity, the NMDA receptor antagonist AP5 was applied to the recording chamber. The frequency of action potentials was not altered, changing from 20 ± 6 to 19 ± 13 spikes/min in the presence of AP5 and back to 22 ± 6/min after washout of AP5 (n = 6; Fig. 3). In contrast to the minor effect of AP5, as indicated in the preceding text, BIC caused a substantial decrease in the frequency of action potentials from 17 ± 3 to 1 ± 0.7/min (n = 21). These data suggest that most action potentials in hypothalamic cells were dependent solely on GABAergic transmission and were independent of the NMDA receptor activation found in hippocampal neurons (Leinekugel et al. 1997; Staley and Mody 1992).

GABA reversal potential is positive to spike threshold

Previous data suggested that GABA-mediated depolarization was based on a relatively high intracellular chloride concentration in the early period of development; when Cl⁻ channels were opened by GABA, Cl⁻ exited the cell (Chen et al. 1996). GABA-mediated depolarization could be inhibitory if it acted to shunt current evoked by another excitatory transmitter or it would be excitatory if it could trigger action potentials. A critical mechanistic question is whether the depolarization induced by GABA would reach or exceed the threshold for the generation of an action potential.

The threshold for the generation of action potentials was measured in 42 hypothalamic neurons (4–7 DIV) recorded with gramicidin perforated patches and ranged from −48 to −21 mV with an average of −34 ± 1 mV. The reversal potential of GABA was determined by applying GABA (10 μM) and recording inward or outward currents at different holding potentials. \( E_{\text{GABA}} = -31 ± 5 \text{ mV} \) (n = 6) in 4–7 DIV hypothalamic neurons. With an extracellular Cl⁻ level of
157 mM, based on the Nernst equation, the estimated concentration of Cl$^{-}$ in the cytoplasm of developing mouse hypothalamic neurons is 45 mM, substantially greater than the 8 mM Cl$^{-}$ reported in mature neurons of rat (Chen et al. 1996). Thus the mean GABA reversal potential was positive to the threshold for spike generation.

To demonstrate that GABA, even at relatively low concentrations, can evoke action potentials under conditions when intracellular Cl$^{-}$ is unperturbed, we used gramicidin-perforated whole cell recording. Several concentrations of GABA were applied to the recorded neuron ($n=21$) during current clamp. As shown in Fig. 4A, the application of GABA induced action potentials even at very low concentrations, including 2, 5, and 10 μM. After the first action potential, there was a plateau of depolarization whose period was dependent on the length and concentration of GABA application. With 10 μM GABA, spikes were found at both the beginning and end of GABA stimulation; during the phase when the membrane potential recovered and returned to a more negative resting membrane potential, firing of one spike or trains of action potentials were observed (Fig. 4A). One micromolar GABA evoked a subthreshold depolarization but no action potential.

In another set of experiments, GABA was applied to the recorded neuron from a micropipette by a brief pressure pulse at a frequency of 1 Hz (Fig. 4D). Each GABA application evoked an action potential. Thus our data here demonstrate that GABA-mediated depolarization could induce action potentials that were phase locked to GABA presence.

To compare the relative ability of GABA and glutamate to evoke action potentials, we used a pair of flow pipes to deliver equimolar concentrations of GABA or glutamate (2 or 10 μM each). Although the different GABA and glutamate ionotropic receptors may have different affinities for their respective ligands, similar concentrations of transmitters were used on the working assumption that roughly equivalent amounts may be released, and to test the relative sensitivities to GABA and glutamate in developing hypothalamic neurons. In young cells, GABA evoked action potentials at these relatively low concentrations in all cells ($n=10$) tested with 2 μM ($n=5$) and 10 μM ($n=5$). In contrast, at 2 μM, glutamate had little effect on membrane potential and did not evoke spikes (Fig. 4B). At 10 μM, glutamate depolarized all five neurons but evoked a spike in only one neuron (Fig. 4C). In all cases,
glutamate evoked a depolarization of smaller amplitude than that evoked by the same concentration of GABA. These data with evoked responses are consistent with our earlier data showing that synaptic release of GABA was similarly more powerful than glutamate in evoking action potentials.

To examine the ionic mechanism of GABA-evoked spikes, Na\(^+\) and Ca\(^{2+}\) channel blockers were used. Cd\(^{2+}\) (100 \(\mu\)M), a wide-spectrum Ca\(^{2+}\) channel blocker, did not block GABA evoked spikes. In contrast, tetrodotoxin (1 \(\mu\)M) completely blocked GABA evoked action potentials (Fig. 5). Similar to what was found in young hypothalamic neurons, the application of BIC depressed the generation of action potentials in developing spinal cord neurons. The frequency of action potentials was decreased from 48 ± 27 to 3 ± 1/min in the presence of BIC (statistically significant, \(P < 0.05\)) and reversed to 37 ± 26/min after washout of BIC. The decrement in the frequency (71.1 ± 12.3%, \(n = 5\) neurons) was very significant (\(P < 0.01\), ANOVA). Application of CNQX and AP5 also caused a reversible reduction in the frequency of action potentials from 42 ± 24 to 23 ± 11/min (\(n = 4\); \(P > 0.05\), ANOVA test). BIC caused a substantially greater reduction in spike frequency than AP5/CNQX (\(P < 0.05\)), suggesting that GABA contributed more in the induction of action potentials than glutamate did in young spinal cord neurons, similar to our findings in hypothalamic neurons.

**GABA drives action potentials in spinal cord neurons**

In early development, depolarizing actions of GABA have been reported in spinal cord neurons (Reichling et al. 1994). In 4–7 DIV spinal cord neurons, gramicidin-perforated whole cell recording was performed under current clamp to test the relative contribution of GABA and glutamate in the induction of action potentials (Fig. 5). Similar to what was found in young hypothalamic neurons, the application of BIC depressed the generation of action potentials in developing spinal cord neurons. The frequency of action potentials was decreased from 48 ± 27 to 3 ± 1/min in the presence of BIC (statistically significant, \(P < 0.05\)) and reversed to 37 ± 26/min after washout of BIC. The decrement in the frequency (71.1 ± 12.3%, \(n = 5\) neurons) was very significant (\(P < 0.01\), ANOVA). Application of CNQX and AP5 also caused a reversible reduction in the frequency of action potentials from 42 ± 24 to 23 ± 11/min (\(n = 4\); \(P > 0.05\), ANOVA test). BIC caused a substantially greater reduction in spike frequency than AP5/CNQX (\(P < 0.05\)), suggesting that GABA contributed more in the induction of action potentials than glutamate did in young spinal cord neurons, similar to our findings in hypothalamic neurons.

**Electrophysiology in brain slices**

The experiments in the preceding text used cultured neurons to show the critical role GABA plays in generating spikes during neuronal development under conditions of random synaptogenesis. To test the hypothesis that synaptic GABA activity plays the same critical role in brain slices, we used whole cell recording and extracellular recording in acute slices. Whole cell recordings were made from mediobasal hypothalamic slices with pipettes containing 29 mM Cl\(^-\), a conservative estimate of intracellular Cl\(^-\) in developing hypothalamic neurons of this age. The baseline frequency of spikes was greater in slices than in cultures. BIC caused a 73.4 ± 15.1% reduction in the frequency of these action potentials, dropping from 36 ± 8 to 13 ± 8/min in the presence of BIC, a statistically significant decrease (\(n = 5\), \(P < 0.05\)).

Extracellular recording is useful in that it does not disturb intracellular ion concentrations, critical for the assessment of GABA’s excitatory actions. An extracellular recording pipette was placed in the arcuate nucleus area of hypothalamic slices and controlled through a motorized micromanipulator. When spike firing was detected, the movement of the recording pipette was stopped. Two developmental ages of hypothalamic slices were studied. One set of slices (P1 group) was obtained from postnatal day 1 to postnatal day 4 mice and the other from P10 mice, which represent different periods of neuronal development. To determine the spike frequency, a 10-min control period was recorded before treatment. The antagonists of GABA and glutamate receptors were given for 10 min, and the spike frequency within the last minute was used as the result of treatment. After 10 min of washout, the last minute of recorded spike frequency was used as the recovery period. In most cases, only a single cell was recorded in each slice. In hypothalamic slices from P1 mice, the frequency of spikes ranged from 0.3

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**FIG. 5.** GABA dominates in the generation of action potentials in young spinal cord neurons (4–7 DIV). A: gramicidin-perforated recording in a 5 DIV neuron from mouse spinal cord. RMP, –52 mV. BIC strikingly reduced the frequency of action potentials (the 2nd trace). In contrast, CNQX (10 \(\mu\)M) and AP5 (100 \(\mu\)M) only caused a moderate reduction in the frequency of action potentials (the 3rd trace). B: bar graph showing that BIC generated a significant decrease (71%) in the frequency of action potentials. CNQX (10 \(\mu\)M) and AP5 (100 \(\mu\)M) only led to moderate reduction in the frequency of action potentials.
to 13 Hz, with a mean of $2.4 \pm 1.4$ Hz ($n = 12$). The amplitude of recorded spikes ranged from 117 to 650 $\mu$V with a mean of $294 \pm 50$ $\mu$V. After a 10-min recording of the baseline, BIC (30 $\mu$M) was bath-applied to the slices. The frequency of spikes was dramatically decreased from $2.4 \pm 1.4$ to $0.3 \pm 0.1$ Hz with the minimal decrease from baseline of 1.3 to 0.9 Hz and the maximal decrease was from a pre-BIC baseline of 13.4 to 0 Hz after BIC treatment (Fig. 6A). In the presence of BIC, the mean percent spike frequency decrease per neuron was significantly decreased to only 27.9 $\pm$ 9.8% of the control ($P < 0.05$, $n = 9$, range from 0 to 73.6% of control; Fig. 6C). In contrast, when the glutamate receptor antagonists CNQX (10 $\mu$M) and AP5 (100 $\mu$M) were applied to hypothalamic slices, the frequency of spikes showed only a modest decrease, from 2.4 $\pm$ 1.1 to 1.7 $\pm$ 0.7 Hz with 88.6 $\pm$ 24.2% of control spikes left ($n = 7$, $P > 0.05$; Fig. 6, A and C).

Parallel experiments were used to examine older slices from P10 mice. The effects of GABA and glutamate receptor antagonists on spike firing frequency were examined (Fig. 6B). In the presence of glutamate receptor antagonists CNQX and AP5, the spike frequency was depressed by $37.3 \pm 13.4\%$ ($n = 4$; $P < 0.05$; Fig. 6D). In contrast, spike frequency showed no decrease ($99.0 \pm 18.7\%$ of control) in the presence of GABA receptor antagonists ($P > 0.5$, $n = 3$; Fig. 6D). We have previously shown that addition of BIC to hypothalamic neurons older than 10 days evoked a strong increase in spike frequency (van den Pol et al. 1998).

Thus the data from hypothalamic slices are consistent with the data from cultures, suggesting that GABA contributed more to spike firing than glutamate in young (P1–P4) brain slices and that this pattern was reversed by postnatal day 10 at which time glutamate served as the primary driving force behind spikes.
We find that in developing brain slices and cultures most action potentials are due not to transmitter-independent spikes nor to glutamate activated excitation but rather are due to GABA release from developing axons. The GABA$_A$ receptor antagonist bicuculline blocks most impulse activity. In contrast, blocking ionotropic glutamate receptors had considerably less effect on spike frequency in early development. We found similar actions of GABA in hypothalamus and spinal cord neurons. In later development, glutamate assumed its well-known role as the primary excitatory transmitter, and GABA took on its inhibitory role in reducing spike generation.

The mechanism for the GABA-mediated spikes appears to be due to the synaptically mediated depolarizing actions of Cl$^{-}$-efflux from the cell, activating voltage-gated Na$^+$ channels. If sufficient GABA is released by local axon terminals to summate enough to drive the depolarization to spike threshold, an action potential occurs. If a low level of GABA is constantly present, this would not be sufficient to drive spikes and may instead serve a shunting function. GABA-mediated depolarization might lead to shunting inhibition as well characterized by Staley and Mody (1995). The core of this shunt concept was that the reversal potential for GABA was positive to the resting membrane potential but negative to the spike threshold potential and that GABA transmission occurred simultaneously with glutamate transmission. In our study, the reversal potential of GABA-mediated responses ($E_{\text{GABA}}$) was measured with undisturbed intracellular chloride concentration by using gramicidin-perforated whole cell recording. $E_{\text{GABA}}$ was positive to the threshold for action potential in developing hypothalamic neurons, allowing GABA to depolarize the membrane potential to the point that an action potential was generated as shown with both synaptic release and flow pipe administration of GABA. Previous reports have shown that bicarbonate ion can also pass through the GABA-gated anion channel (Kaila et al. 1993). We found that GABA-mediated activation of spikes has no bicarbonate dependence and occurs even in HEPES buffer in the relative absence of bicarbonate.

Intracellular Cl$^{-}$ was about four to five times greater in developing than in mature neurons, suggesting an active pump or transporter is moving Cl$^{-}$ into the developing cell, perhaps a cation/Cl$^{-}$ cotransporter moving Cl$^{-}$ inward, or that a Cl$^{-}$ transporter that is outward in mature neurons may have a reversed polarity in developing neurons. In addition, many outward Cl$^{-}$ transporters are relatively inactive during early development (Clayton et al. 1998; Lu et al. 1999; Luhmann and Prince 1991; Rivera et al. 1999; Staley et al. 1996). One mechanism that may explain GABA’s stronger role than glutamate in development is that GABA receptors develop earlier than glutamate receptors in the hypothalamus (Chen et al. 1995; van den Pol et al. 1995) and spinal cord (Walton et al. 1993), and, as we demonstrate here, GABA application can evoke spikes at a developmental stage when an equimolar glutamate concentration does not even depolarize neurons. GABA depolarizing actions begin early in development as GABA is released from advancing axon growth cones even before synaptic contact is established (Gao and van den Pol 2000). That GABA evolves from an excitatory to inhibitory transmitter during neuronal development in cultures, as it does in the brain, suggests that the negative shift in $E_{\text{Cl}^-}$ may either be an intrinsic property of developing neurons or that the necessary factors generating the developmental shift are available in vitro.

In a number of seminal papers focusing on hippocampal neurons in vitro and in slices, GABA was shown to play a critical role in generating giant depolarizing potentials (Ben-Ari et al. 1989; Cherubini et al. 1990, 1991; Leinekugel et al. 1997). This is based on a mechanism where GABA relieved the NMDA receptor of its Mg$^{2+}$ block, resulting in glutamate-mediated giant depolarizing potentials. Blockade of the NMDA receptor eliminated the ability of GABA to elicit giant depolarizing potentials (Leinekugel et al. 1997). Thus GABA and glutamate have equivalent and synergistic roles during this stage of hippocampal development. Our preliminary results with hippocampal cells are consistent with these previous published observations. But only in hippocampal cells did glutamate play a substantial role in spike generation in developing cells; this was not true in neurons of the two CNS regions examined in the present study, hypothalamus and spinal cord. In hypothalamic neurons in vitro and in slices, the blockade of glutamate transmission with CNQX and AP5 did not depress the majority of the action potentials, and by itself the NMDA receptor antagonist AP5 had little effect on the frequency of action potentials. Together these results suggest that hippocampal cells in this context may not necessarily be representative of other brain regions. One mechanism that may account for this difference is the high level of expression of glutamate receptors, particularly the NMDA receptor, in the developing hippocampus (Tremblay et al. 1988). Another factor that may account for the strong role of GABA in the hypothalamic neurons compared with the hippocampal pyramidal neurons is that hypothalamic neurons are smaller and have smaller dendritic arbors and a higher input resistance (van den Pol et al. 1990), which would increase the probability that low levels of GABA would evoke spikes. Thus GABA plays a bigger role than glutamate in driving long distance signaling via action potentials in early hypothalamic development.

Another critical factor is the GABA reversal potential. In developing hippocampal cells, $E_{\text{GABA}}$ was suggested to be about $-51$ mV (Cherubini et al. 1990), whereas in hypothalamic neurons of similar age in the present study $E_{\text{GABA}}$ was $-31$ mV. GABA would generate greater depolarizing actions in the presence of the more positive $E_{\text{GABA}}$.

A number of interesting studies have demonstrated that the excitatory actions of glutamate can play a profound role in modulating the formation of connections in the developing brain (Hofer and Constantine-Paton 1994). Enhancement of synaptic stabilization through Hebbian mechanisms has focused on glutamatergic synapses, and the role of GABA has been relegated to some degree to the modulation of the glutamate synapse (Ben-Ari et al. 1997). In hippocampal neurons, GABA-mediated long-term depression was dependent on both GABA and NMDA receptors (Caillard et al. 1999). The concept of a Hebbian synapse has been applied primarily to glutamate synapses, leaving the other half of the synapses in the brain, the GABA synapses, as a mechanistic mystery in terms of activity-dependent synapse stabilization. As our data indicate that axonal release of GABA routinely evokes spikes in neurons from some regions of the developing brain, perhaps the concept of a Hebbian synapse can apply to GABA synapses independent of glutamate activity.
GABA MEDIATES SPIKE ACTIVITY IN DEVELOPING NEURONS

In conclusion, GABA appears to play a more powerful role than glutamate in generating spikes during early development in some brain regions. Thus many aspects of neuronal development in these regions can be modulated by activity including synaptic stabilization, axonal pathfinding or pruning, growth cone orientation, and elaboration of functional efficacious circuitry may be regulated by GABA in early neuronal development, and by glutamate later in development.

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