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GABA Neurotransmission in the Hypothalamus: Developmental Reversal from Ca^{2+} Elevating to Depressing

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GABA is the primary inhibitory transmitter of the adult hypothalamus, synthesized by many neurons and found in 50% of the presynaptic boutons. GABA causes a decrease in Ca^{2+} in mature hypothalamic neurons *in vitro* by depressing cellular activity through opening Cl^- channels. Despite the early expression of $GABA_A$ receptors in the embryonic hypothalamus (E15), the cellular function of GABA in the developing hypothalamus has received little attention. In the present study the role of GABA in modulating intracellular Ca^{2+} in developing hypothalamic neurons was studied with fura-2 digital imaging.

GABA (0.5–500 μM) applied to embryonic hypothalamic neurons elicited a dramatic and rapid increase in intracellular Ca^{2+} This Ca^{2+} rise could be completely blocked by the $GABA_A$ antagonist bicuculline (20 μM) and persisted in the presence of tetrodotoxin (1 μM). The Ca^{2+} elevation induced by GABA was greater than that of equimolar concentrations of the excitatory transmitter glutamate in early development. The number of E15 neurons that responded to GABA with a Ca^{2+} rise increased during the first few days of culture, reaching 78% after 4 d *in vitro*. The Ca^{2+} rise was 87% blocked by cadmium (100 μM) and 85% blocked by nimodipine (1 μM), indicating that the mechanism of Ca^{2+} increase was primarily via L-type voltage operated Ca^{2+} channels.

Addition of bicuculline to synaptically coupled cultures caused a significant decrease in Ca^{2+} 4–10 d after culturing, indicating hypothalamic neurons were secreting GABA at an early age of development, and that sufficient GABA was released to elicit an increase in Ca^{2+}. This effect was seen even after blocking all glutamatergic activity with glutamate receptor antagonists. In contrast, GABA elicited no Ca^{2+} rise in older neurons (>18 d in vitro), and the action of bicuculline reversed and caused a large increase in Ca^{2+} in spontaneously active neurons. Similar findings were obtained in cultures enriched in GABAergic neurons from the suprachiasmatic nucleus. To determine if the Ca^{2+} stimulating role of GABA on developing neurons was restricted to the hypothalamus and a few other regions, or whether it might exist throughout the brain, we examined the Ca^{2+} responses in cultured olfactory bulb, cortex, medulla, striatum, thalamus, hippocampus, and colliculus. The majority (75%) of developing neurons from each region showed a Ca^{2+} rise in response to GABA.

Together these data suggest that GABA elevates Ca^{2+} in developing, but not mature, neurons from the hypothalamus and all other brain regions examined. As Ca^{2+} plays a crucial role in modulating gene expression, enzymatic function, neurite outgrowth, and transmitter release, GABA may serve as an excitatory intercellular messenger involved in developmental signalling prior to the time when its primary function is to inhibit neuronal activity.

[Key words: neuroendocrine, bicuculline, $GABA_A$ receptor, chloride, glutamate]

During brain development, the amino acid transmitter GABA may modulate neuronal function and growth. GABA increases neurite outgrowth of brain and retinal neurites (Spoerri, 1988; Michler, 1990; Barbin et al., 1993) and modulates synapse formation of cultured cells (Meier et al., 1984; Hansen et al., 1987). Although GABA directly opens chloride channels, GABA can influence the levels of intracellular Ca^{2+} by influencing voltage activated Ca^{2+} channels. Ca^{2+} exerts a wide variety of effects on the developing brain, where it can modulate the rate and direction of neuritic growth (Mattson and Kater, 1987) and influence gene expression (Vaccarino et al., 1992; Bading et al., 1993).

GABA appears to be the primary inhibitory transmitter in the adult hypothalamus. Immunocytochemical studies with the antisera against GABA or against the GABA synthesizing enzyme glutamate decarboxylase (GAD) show strong staining in cell bodies and axons throughout the rat hypothalamus (Tappaz et al., 1982; van den Pol, 1985, 1986; van den Pol and Tsujimoto, 1985). Nearly 50% of all presynaptic boutons in the hypothalamus are immunoreactive with GABA, as studied quantitatively with postembedding immunogold ultrastructural immunocytochemistry (Decavel and van den Pol, 1990); of the hundreds of neurons studied with electron microscopy, all had a GABAergic input (Decavel and van den Pol, 1990, 1992). Physiologically, application of GABA in the adult hypothalamus leads to neuronal inhibition mediated by opening Cl^- channels, and application of $GABA_A$ receptor antagonists greatly reduce or eliminate IPSPs (Randle et al., 1986; Kim and Dudek, 1990; Nissen and Renaud, 1994).

In the course of studying the development of glutamate mediated Ca^{2+} excitability in hypothalamic neurons (Obrietan and van den Pol, 1995; van den Pol et al., 1995), we found some unusual neurons in young cultures that rather than showing the

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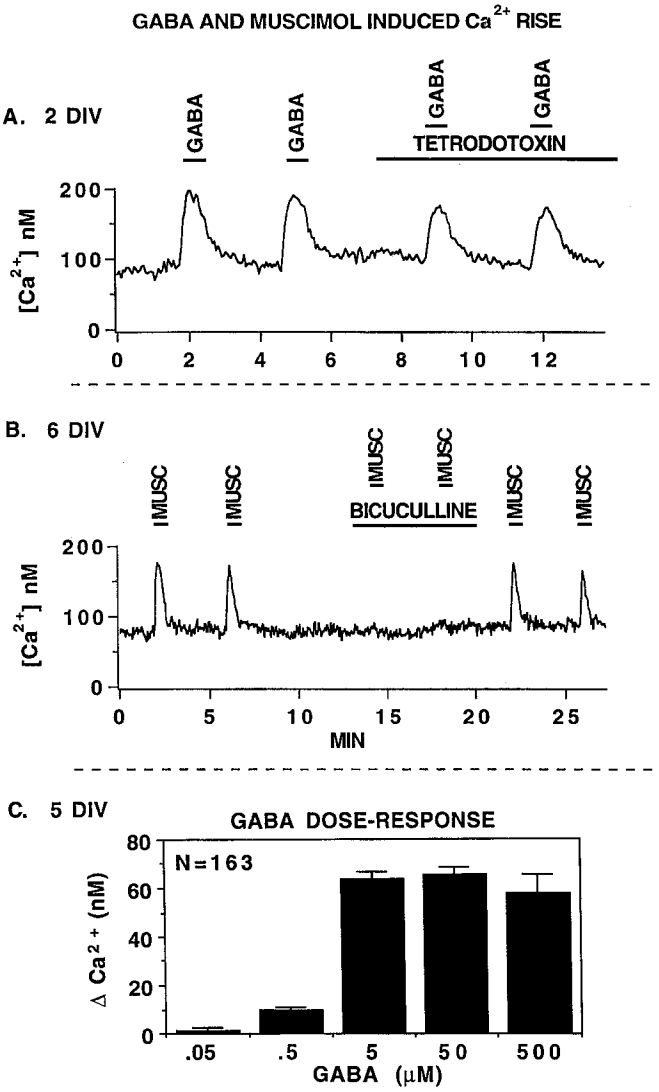

Figure 1. A, After 2 d *in vitro* (2 DIV) embryonic day 18 (E18) hypothalamic neurons were exposed to 20 μM GABA. Ca^{2+} levels increased and decreased immediately with the repeated addition and removal of GABA. Even in the presence of 1 μM tetrodotoxin (TTX), GABA was still capable of increasing Ca^{2+} levels. The response of this neuron is characteristic of all neurons assayed. *B*, A typical Ca^{2+} rise in response to 5 μM muscimol is shown; 6 DIV. *C*, The Ca^{2+} responses of neurons to different concentrations of GABA are plotted. 5 DIV.

expected decrease in Ca^{2+}, showed an increase when GABA was added. Previous work with nonhypothalamic neurons had suggested that GABA may exert a transient depolarizing influence on developing neurons (Connor et al., 1987; Ben-Ari et al., 1989; Yuste and Katz, 1991), suggesting that if GABA had a similar effect on developing hypothalamic neurons, a rise in intracellular Ca^{2+} might result. The present study examines the role of GABA in elevating Ca^{2+} in young hypothalamic neurons, but decreasing Ca^{2+} in older hypothalamic neurons.

Materials and Methods

Tissue culture. The hypothalamus was removed from embryonic Sprague–Dawley rats, stripped of meninges, and washed three times in standard tissue culture medium, and then incubated in an enzymatic solution (10 U/ml papain, 500 μM EDTA, 1500 μM $CaCl_2$, 0.2 mg/ml L-cysteine in Earl's balanced salt solution). Embryonic day 15 (E15) tissue was incubated for 20 min in the protease solution, E18 tissue was treated for 30 min. The tissue was then pelleted by centrifugation, papain solution removed by aspiration, and tissue mechanically triturated and pelleted three times to generate a single cell suspension. The cell suspension was then plated onto 22 mm square coverslips that had been washed in a mild soap solution, rinsed and autoclaved and coated with high molecular weight poly-L-lysine (540,000 Da; Collaborative Research).

To ensure high local neuronal density, cells were plated within a 7 mm diameter glass ring placed on top of the coverslip; 45 min after plating the ring was removed. Most of the cells adhered to the coverslip surface within 10 min of plating. Cultures were maintained at 37°C and 5% CO_2 in a Napco 5410 incubator in glutamate- and glutamine-free MEM (GIBCO) supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, and 6 gm/liter glucose.

To limit the proliferation of non-neuronal cell types, cytosine arabinofuranoside (1 μM) was added to the tissue culture medium 4 d after plating and maintained until 14 d *in vitro* (14 DIV). To maintain high density neuronal cultures the glutamate receptor antagonists CNQX (10 μM) and AP5 (100 μM) were added to the tissue culture medium at 4 DIV. CNQX and AP5 enhance long term survival of hypothalamic cultures through the inhibition of glutamate mediated excitotoxicity (Choi 1987; Furshpan and Potter, 1989; Obrietan and van den Pol, 1995). Medium was changed twice a week.

The region of the suprachiasmatic nuclei (SCN) was dissected from E18 brains. Due to the small size of the SCN at E18, these cultures contained neurons not only from the SCN, but also from the immediate surrounding anterior hypothalamus. These cells were treated to an identical digestion, plating, and feeding protocol as that described for hypothalamic cultures.

Calcium digital imaging. Prior to the beginning of the experiment, cells were incubated in standard HEPES perfusion solution (137 mM NaCl, 25 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 3 mM $CaCl_2$, pH 7.4) containing 5 μM fura-2 acetoxymethyl ester (Molecular Probes) for 20 min at 37°C. To inhibit endogenous synaptic activity during fura-2 loading, AP5 and CNQX were added to the incubation solution of cells cultured longer than 18 d. The cells were then washed and allowed to recover for 15 min prior to the start of the experiment. Coverslips were then loaded into an eight port 180 μl microscope perfusion chamber (Forscher et al., 1987) and perfused at a constant rate of 1 ml/min. Solutions moved as a straight wave across the chamber allowing for rapid application and removal of receptor agonists and antagonists. Cells were imaged using a 40× Olympus objective with high 340/380 nm transmittance on a Nikon Diaphot 300 inverted microscope. Neurons were identified by their responsiveness to the application of NMDA and by their phase-bright appearance. After 2 DIV neurons had extended long dendritic and axonal processes and were usually found growing on top of an astrocyte monolayer. Ca^{2+} digital recordings were made from the cell soma. All experiments were performed at room temperature. To examine NMDA responses, glycine (2 μM) was added to perfusion solutions that contained 0 Mg^{2+} (Nowak et al., 1984) to enhance NMDA receptor activity (Johnson and Ascher, 1987).

Data were recorded and all peripheral devices were controlled by a Universal Imaging 486 computer with FLUOR software. Calibrated Ca^{2+} values from up to 64 cells could be recorded simultaneously. Calibrations of Ca^{2+} were performed as described by Grynkiewicz et al. (1985) with Ca^{2+} standards and fura-2 free acid from Molecular Probes. Excitation light from a 150 W xenon lamp powered by an Optiquip transformer was filtered through a Sutter filter wheel driven by a Lambda-10 microprocessor. Attenuation of excitation light by 90% was accomplished with neutral density filters allowing for long recording periods without any sign of phototoxicity; 16 video frames of data were recorded from both wavelengths every two seconds. Ca^{2+} data from single cells were transferred to an Apple 840AV computer and analyzed with IGOR PRO software (WaveMetrics). Responses to the addition of excitatory amino acids are reported as the mean Ca^{2+} rise from basal Ca^{2+} levels ± the standard error of the mean.

Cytosine arabinofuranoside, GABA, glycine, and glutamate were purchased from Sigma, AP5, CNQX, bicuculline, and TTX were purchased from Research Biochemicals International. Papain was purchased from Worthington Biochem.

Results

GABA induced rises in intracellular Ca^{2+}

The addition of 20 μM GABA to E18 hypothalamic neurons cultured for 2 d caused an immediate and reproducible rise in

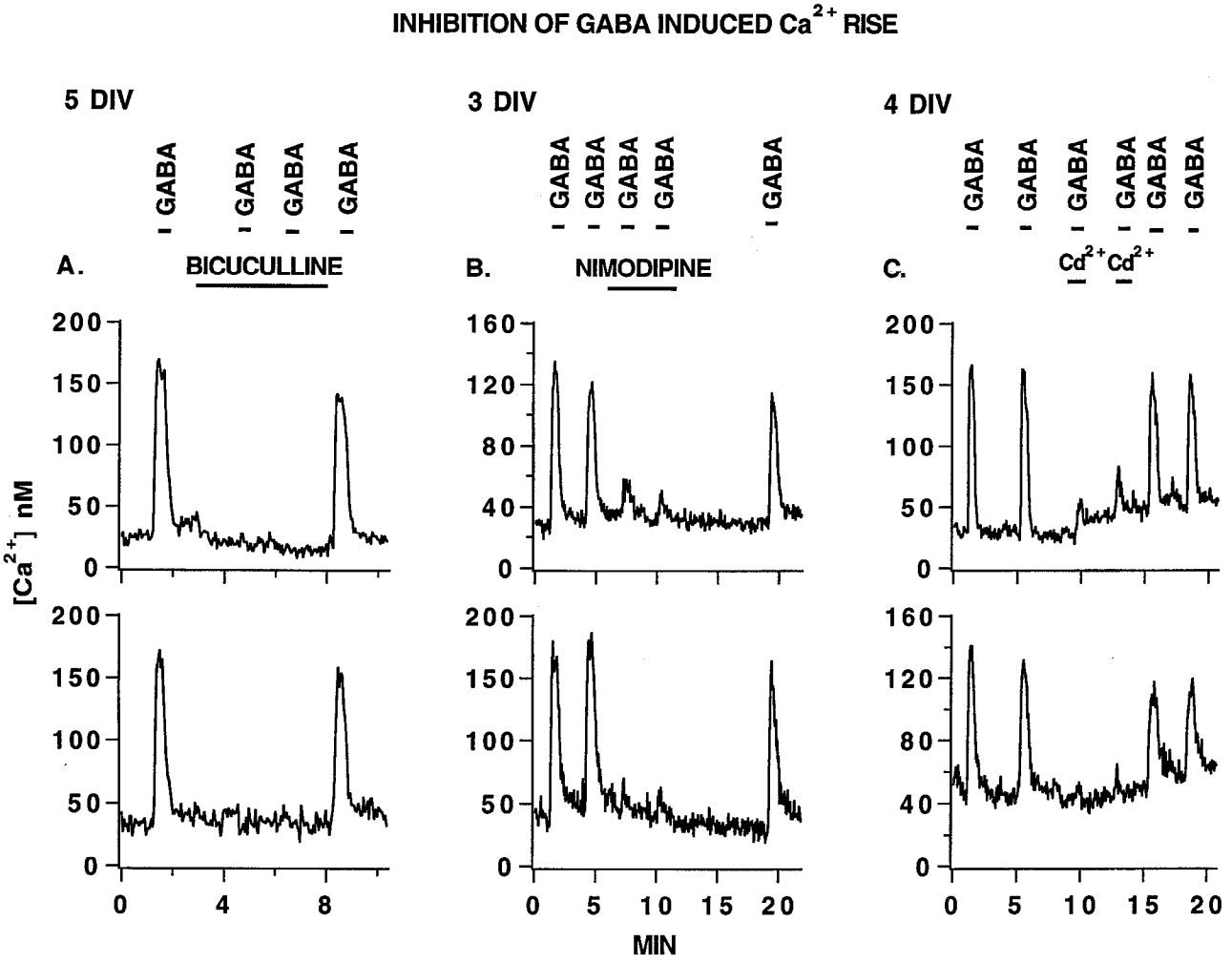

Figure 2. Inhibition of GABA induced Ca^{2+} rises. *A*, The addition of 20 μM bicuculline to the perfusion solution inhibited GABA induced Ca^{2+} increases in a reversible manner. 5 DIV. *B*, 1 μM nimodipine reversibly suppressed the Ca^{2+} rise, indicating the involvement of L-type voltage activated Ca^{2+} channels. 3 DIV. *C*, The addition of 100 μM Cd^{2+} also inhibited Ca^{2+} increases. Due to its toxic effects if used for long durations, Cd^{2+} was applied just prior to and after the removal of 20 μM GABA. 4 DIV.

intracellular Ca^{2+} levels (Fig. 1*A*). GABA washout quickly returned the neurons to resting Ca^{2+} levels. GABA also increased Ca^{2+} levels in the presence of 1 μM tetrodotoxin (Fig. 1*A*), suggesting that secondary synaptic activity is not the cause of the Ca^{2+} rise. The Ca^{2+} rise elicited by the addition of GABA could be completely inhibited by the $GABA_A$ receptor antagonist bicuculline (Fig. 2*A*). GABA (5 μM) caused a mean Ca^{2+} rise of 89.8 ± 8.6 nM (SEM), whereas 5 μM GABA in the presence of 20 μM bicuculline induced a negligible mean rise (1.3 ± 1.3 nM) in 42 neurons. This suggests that the GABA induced Ca^{2+} rise is initiated by the activation of the $GABA_A$ and not the $GABA_B$ receptor. Ca^{2+} rises could also be induced by the addition of the $GABA_A$ receptor agonist muscimol (Fig. 1*B*). Stimulation with 5 μM muscimol increased Ca^{2+} in 77% of 128 E18 neurons *in vitro* for 6 d, with a mean Ca^{2+} rise of 65 ± 5 nM. GABA-induced Ca^{2+} rises could be inhibited by the addition of the L-type Ca^{2+} channel blocker nimodipine (Fig. 2*B*). Nimodipine (1 μM) reduced the Ca^{2+} increase by an average of 86% in 50 neurons. Similarly, the addition of 100 μM Cd^{2+} (Fig. 2*C*) inhibited GABA induced Ca^{2+} rise by 87% in 35 neurons tested. Ca^{2+} rises could be evoked using as little as 500 nM GABA. The effects of GABA were maximal at 5 μM and did not increase significantly with higher GABA concentrations up to 500 μM (Fig. 1*C*).

Modulation of Ca^{2+} levels by endogenous release of GABA

Baseline Ca^{2+} levels in cultured hypothalamic neurons can be reduced by the addition of the glutamate receptor antagonists AP5 and CNQX (van den Pol and Trombley, 1993). Figure 3, *A1–A3*, are examples of this from E18 cultures after 6 DIV; the removal of AP5/CNQX from the perfusion solution increased basal Ca^{2+} levels. The addition of bicuculline to the neuron in Figure 3*A3* had virtually no effect on basal Ca^{2+} levels, whereas the Ca^{2+} levels of the neurons shown in Figure 3, *A2* and *A3*, were depressed by the addition of bicuculline. To determine whether GABA was being released by hypothalamic cells, bicuculline was added to neurons constantly perfused with AP5/CNQX to block glutamate receptor activity. The addition of bicuculline reversibly depressed basal Ca^{2+} levels (Fig. 3*B*) in 37% of 128 neurons tested. These findings suggest that in early hypothalamic development GABA is actively secreted and that it increases intracellular Ca^{2+}.

In the CNS GABA is primarily thought of as an inhibitory neurotransmitter. Contrary to this, early in hypothalamic devel-

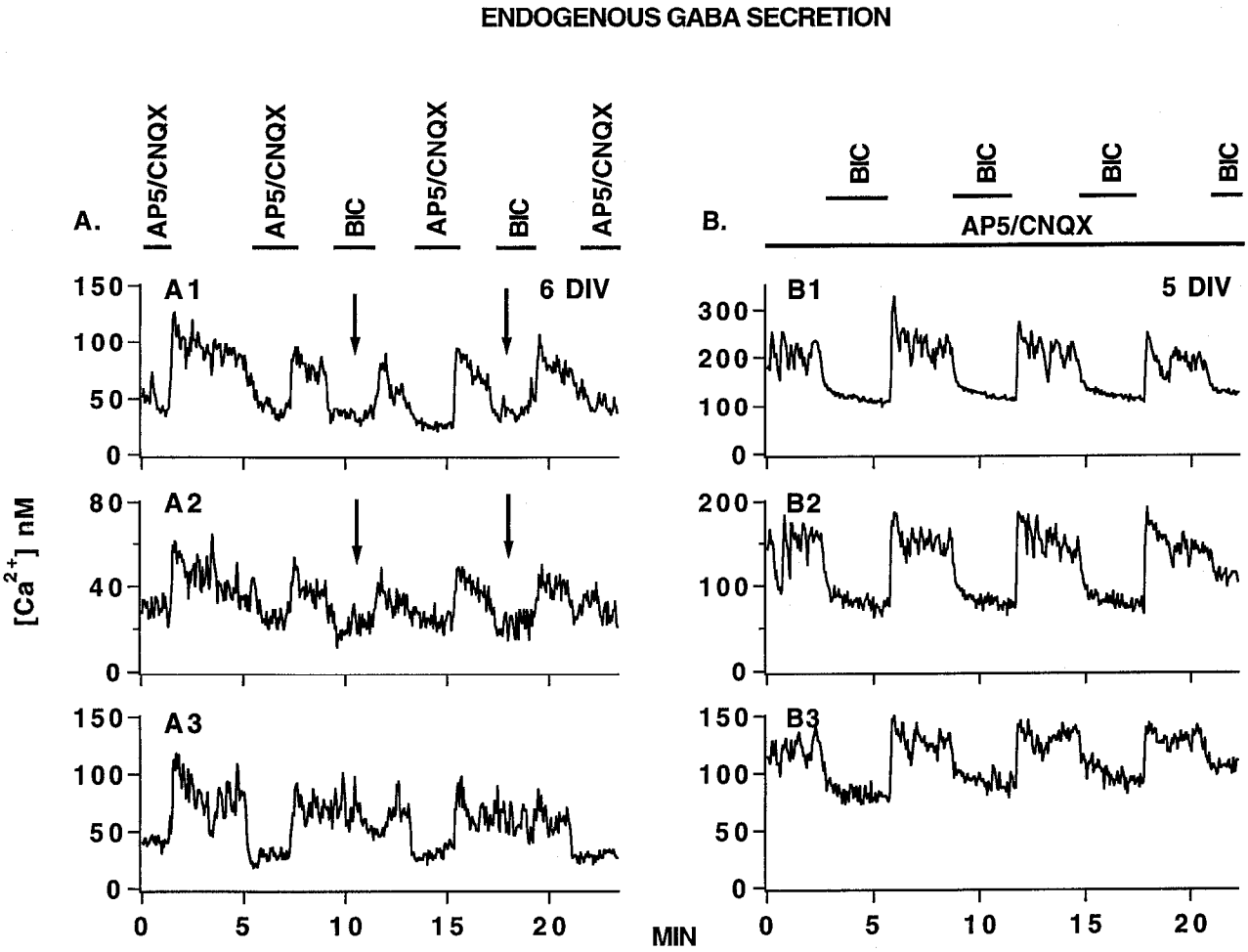

Figure 3. *A*, Endogenous glutamatergic activity was visible with the removal of the glutamate receptor antagonists AP5 (100 μM) and CNQX (10 μM) from the 0 Mg^{2+} perfusion solution. *A3* shows a neuron that increases Ca^{2+} levels with the removal of glutamate receptor agonist. The elevated Ca^{2+} level was unaffected by the addition of the $GABA_A$ receptor antagonist bicuculline (20 μM). Unlike *A3*, the addition of bicuculline depressed elevated Ca^{2+} levels in the neurons *A1* and *A2* (*arrows*). All cells are cultures of E18 hypothalami. 6 DIV. *B*, Neurons were perfused in the constant presence of AP5 (100 μM) and CNQX (10 μM). Basal Ca^{2+} levels of all three neurons were reversibly depressed by the addition of bicuculline (20 μM). 5 DIV.

opment GABA may function as a Ca^{2+} elevating neurotransmitter. We examined the developmental time point at which $GABA_A$ receptors switch roles from Ca^{2+}-elevating to Ca^{2+}-depressing. Figure 4 shows neurons from three developmental ages treated to the identical experimental protocol. At 8 DIV (Fig. 4*A*) the addition of 20 μM bicuculline depressed Ca^{2+} levels and Ca^{2+} fluctuations. At 13 DIV (Fig. 4*B*), the role of the $GABA_A$ receptor had changed. The application of bicuculline dramatically increased Ca^{2+} in all three neurons. Similar results were seen in neurons cultured for 33 d (Fig. 4*C*). These data indicate that during the period between 8 and 13 DIV the $GABA_A$ response changed from Ca^{2+}-elevating to Ca^{2+}-depressing.

Figure 5 shows the effects of bicuculline on unblocked, basal Ca^{2+} levels. From 2 DIV through 13 DIV, bicuculline depressed Ca^{2+} levels. The percentage of neurons showing reductions in Ca^{2+} with bicuculline increased dramatically from 2 DIV (2%) to 4 DIV (25%), reaching a maximum at 8 DIV (nearly 40%). After 8 DIV the percentage of neurons depressed by bicuculline dropped dramatically. By 13 DIV only 4% of the neurons responded, and by 25 d the effect was completely lost. In contrast, bicuculline's ability to increase neuronal Ca^{2+} levels started very low (2% at 8 DIV) and increased steadily throughout time *in vitro*, reaching a maximum of 97% at 25 DIV. The changing role of the $GABA_A$ receptor is clearly visible at 10 DIV. At this time point bicuculline increases and decreases Ca^{2+} levels in nearly equal numbers of neurons on the same coverslip. Data collected prior to 10 DIV show the $GABA_A$ receptor playing a predominately Ca^{2+} elevating role. After 10 DIV activation of the $GABA_A$ receptor depressed Ca^{2+}.

Transition of GABA from excitatory to inhibitory activity

The excitatory effects of GABA diminish by 13 DIV (Fig. 6). In all three neurons, excitatory synaptic activity initiated by the removal of AP5/CNQX from the perfusion solution was inhibited by the addition of GABA. Unlike Figure 1 where in the presence of TTX, GABA induced a Ca^{2+} rise in the majority of 2 DIV neurons (71% of 64 neurons) GABA induced a Ca^{2+} rise in only 17% of 110 neurons *in vitro* for 13 d.

GABA's transition from Ca^{2+}-elevating to Ca^{2+}-depressing neurotransmitter temporally corresponds with the development of complex glutamatergic neurotransmission. Of interest was the finding that bicuculline potentiated endogenous glutamatergic activity primarily in neurons displaying synchronized Ca^{2+} spikes. Neurons (10 DIV) in Figure 7, *A* and *B*, exhibited syn-

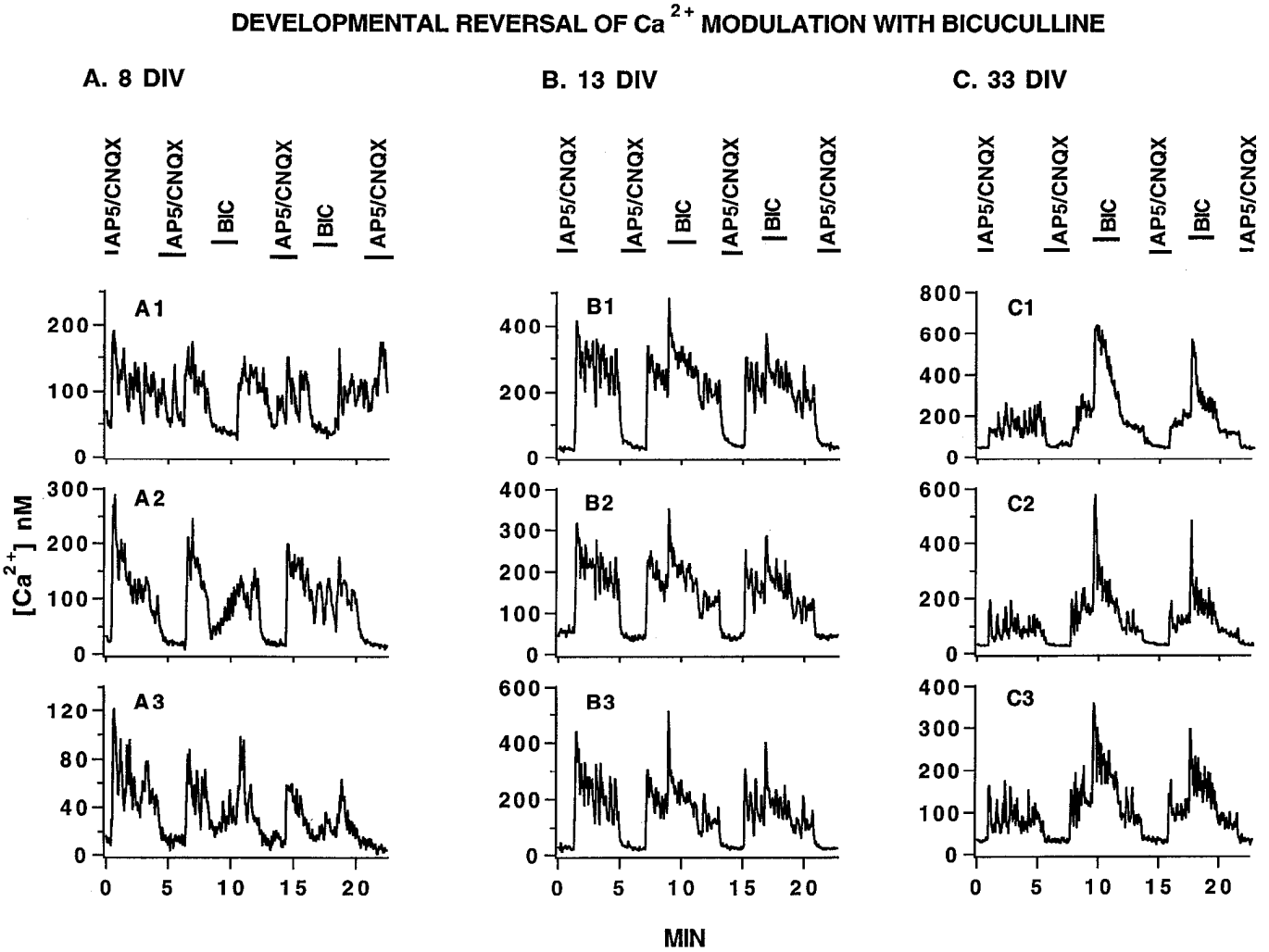

Figure 4. The effects of bicuculline on Ca^{2+} levels reversed for E18 hypothalamic neurons between 8 and 13 DIV. *A*, 8 DIV neurons were removed from glutamate receptor blockade [AP5 (100 μM) and CNQX (10 μM)] in a O Mg^{2+} perfusion solution. The application of 20 μM bicuculline to unblocked neurons depressed Ca^{2+} levels. Ca^{2+} levels in *A1* were not affected by the removal of glutamate receptor blockers. *B*, 13 DIV neurons were treated to the identical protocol used for 8 DIV neurons. Of interest is the reversed effect of bicuculline on Ca^{2+} levels; bicuculline reproducibly increased Ca^{2+} levels in all three neurons. The effects of bicuculline on 33 DIV neurons (*C*) were identical to those found for 13 DIV neurons.

chronized Ca^{2+} spikes with the removal of AP5/CNQX from the perfusion solution. Endogenous activity was potentiated (larger spikes and increased Ca^{2+} level) by the application of bicuculline. The Ca^{2+} level in neuron C was elevated by the removal of AP5/CNQX but, unlike neurons A and B, the Ca^{2+} spikes were not synchronized. When bicuculline was applied to neuron C, the basal Ca^{2+} level was depressed. The neuron in Figure 7*D* was unaffected by the removal of AP5/CNQX but the addition of bicuculline reduced basal Ca^{2+} levels. Of 27 neurons (studied simultaneously with digital imaging) with complex, glutamate-mediated synchronized activity, 81% had Ca^{2+} levels potentiated and 19% had Ca^{2+} levels depressed by bicuculline. Of 35 neurons not exhibiting glutamate-mediated synchronized activity, 14% showed potentiation and 86% showed depression of Ca^{2+} levels.

Comparison of GABA, glycine, and glutamate induced Ca^{2+} responses during development

To compare the Ca^{2+} responses to GABA with other amino acids at different developmental ages, E15 neurons were stimulated with 20 μM GABA, 20 μM glutamate, or 20 μM glycine. Figure 8 shows examples of characteristic cells from three different durations *in vitro*. In Figure 8*A* neurons were plated 4 hr prior to imaging. The neuron in Figure 8*A1* had nearly equivalent, reproducible Ca^{2+} increases to the application of GABA and glutamate and a slightly less robust response to glycine. In Figure 8*A2* glutamate induced a much larger Ca^{2+} rise than GABA, whereas in Figure 8*A3*, the converse was true. A similar heterogeneity of reproducible responses was seen in Figure 8*B*. Neurons from 12 DIV (Fig. 8*C*) had a notable absence of strong GABA induced Ca^{2+} increases. All cells that showed a Ca^{2+} rise in response to glycine application also responded to GABA. A subpopulation of neurons were found that responded only to GABA and not to glycine.

Several interesting trends appeared when peak Ca^{2+} responses from each agonist were combined into mean responses for each time point (Fig. 9*A*). As was the case with bicuculline's ability to depress basal Ca^{2+} levels [percentage of neurons increased over the first 8 DIV, then decreased slowly (Fig. 5)] GABA's ability to increase Ca^{2+} levels started at low amplitudes (12.1 ±

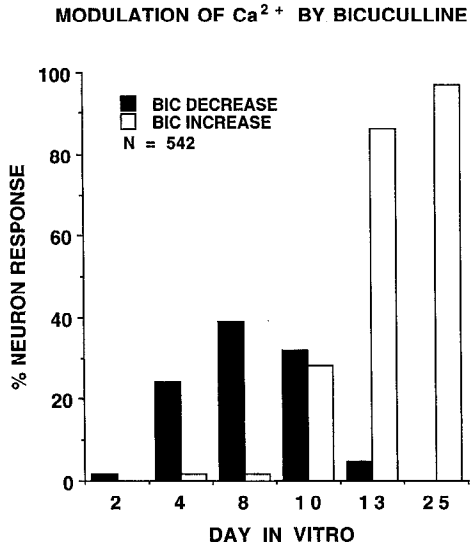

Figure 5. The ability of bicuculline (20 μM) to either decrease or increase Ca^{2+} levels of E18 hypothalamic neurons was plotted from 2 to 25 DIV. From 4 to 8 DIV the predominant effect of bicuculline was to decrease Ca^{2+} levels. By 10 DIV, Ca^{2+} levels in roughly equal numbers of neurons were either increased or decreased by bicuculline. At 13 DIV the effect was dominated by potentiation and by 25 DIV the sole response to bicuculline was an increase in Ca^{2+}.

1.5 nM at 0 DIV), gradually increased to a mean rise of 77.5 ± 4.5 nM by 4 DIV, and subsequently diminished. In contrast, glutamate-induced responses start out low (13.9 ± 2.4 nM at 0 DIV) and continue to rise throughout the time tested. GABA induced a larger rise after 1 and 2 DIV than did glutamate. At 4 DIV mean GABA response levels peaked, whereas glutamate response values continued to rise. At 16 DIV the response generated by the application of glutamate was over 14 times greater than that induced by GABA. Similar to glutamate, 55 mM K^+ induced Ca^{2+} rises increased developmentally. Glycine was the least effective agonist, only slightly increasing Ca^{2+} levels during early development.

The percentage of neurons that responded to each agonist is depicted in Figure 9*B*. A similar pattern to that seen in Figure 9*A* developed. The percentage of cells responsive to the application of GABA rose steadily from 18% at 0 DIV up to 78% by 4 DIV and thereafter fell. The percentage of glutamate responsive cells increased throughout the time in culture, reaching a plateau at 98% at 12 DIV. The percent of glutamate responsive cells lagged behind GABA responsive cells (the number of GABA responders was nearly twice that of glutamate responders at 1 DIV) up to 3 DIV. Cells that responded to 55 mM K^+ increased through time in culture, reaching 100% by 12 DIV. Glycine responsive cells followed the same general trend as GABA responsive cells; a slow rise to a peak at 4 DIV and declining thereafter.

To study the combined effect of GABA and glutamate in young hypothalamic neurons, E18 4 DIV cultures were stimulated with 5 μM GABA, 5 μM glutamate, or a combination of 5 μM GABA and 5 μM glutamate. In some neurons the response to glutamate was greater, and in others the response to GABA was greater. No neurons were found that showed a decrease in Ca^{2+} in response to either amino acid. The mean Ca^{2+} rise in response to the application of GABA was 55 ± 3.2 nM. The mean Ca^{2+} response to glutamate was 101 ± 5.3 nM. Of interest was the finding that the mean Ca^{2+} response to the combined addition of agonists (79 ± 3.6 nM) was intermediate between the responses to GABA and glutamate (N = 119). Of the 119 neurons tested, 91% had a greater response to either GABA or glutamate than to the response elicited by the combined addition of the agonists. The response of the remaining 9% was slightly greater than the largest response to either glutamate or GABA, but in only 4% of the neurons was the combined Ca^{2+} rise statistically greater (>2 × the SEM).

GABA in the suprachiasmatic nucleus

Whereas the hypothalamus in general has a high density of GABAergic neurons, the suprachiasmatic nucleus (SCN), the circadian clock of the mammalian brain, is composed almost entirely of GABA immunoreactive cells (Card and Moore, 1984; van den Pol and Tsujimoto, 1985; Okamura et al., 1989). The region of the SCN was cultured on E18 and treated to similar protocols as hypothalamic cultures. Histological analysis confirmed that the SCN and the immediately surrounding hypothalamic area were included in these cultures. Figure 10*A* shows three neurons from these cultures that responded to the application of GABA with a rise in basal Ca^{2+} levels, both in the absence and presence of TTX. Figure 10*B* shows 4 DIV neurons responding to multiple applications of GABA, glutamate and glycine. Both the mean response and the number of responsive neurons were compared at 4 DIV and 18 DIV; 128 neurons were tested at each time point. Over time in culture, an increased mean Ca^{2+} rise was found with the applications of glutamate (69 ± 4 nM at 4 DIV and 283 ± 8 nM at 18 DIV) and K^+ 55 mM (180 ± 6 nM at 4 DIV and 283 ± 8 nM at 18 DIV). In contrast a decreased mean response was found with GABA (39 ± 3 nM at 4 DIV and 13 ± 2 nM at 18 DIV) and glycine (14 ± 3 nM at 4 DIV and 5 ± 1 nM at 18 DIV). Similarly, the total number of neurons with a Ca^{2+} rise greater than 30 nM changed with time in culture. At 4 DIV 46% of the cells responded to GABA with a Ca^{2+} rise, 81% responded to glutamate, 4% to glycine, and 96% to K^+. By 18 DIV 13% of the cells responded to GABA with a Ca^{2+} rise, 100% responded to glutamate, 1% to glycine, and 100% to K^+. Inhibition of the $GABA_A$ receptor by bicuculline after 6 DIV (in the constant presence of AP5/CNQX) depressed basal Ca^{2+} levels in 50% of the 128 neurons tested. This 50% was the largest number of neurons that responded to bicuculline with a Ca^{2+} depression at any time during development, either in whole hypothalamus or SCN-enriched cultures. This indicated that in these SCN enriched cultures with blocked glutamate receptors, GABA secreted from cells in the culture elevated cytoplasmic Ca^{2+} of many neurons.

Other brain regions

To determine if GABA (30 μM) would induce Ca^{2+} rises only in a few regions of the brain, or whether its effects were more universal on developing neurons, we compared the responses of 3 DIV hypothalamic cultures to cultures from seven other brain regions from E18 rats. The majority (75%) of neurons from each region showed GABA-elicited Ca^{2+} rises. This was based on a total 508 neurons, with 64 from each region except hypothalamus which had 60 neurons. The percent of neurons showing a rise of at least 30 nM in each area was as follows: hypothalamus (61%), cortex (89%), hippocampus (83%), thalamus (64%), medulla (76%), colliculus (83%), striatum (72%), and olfactory bulb (61%). The mean Ca^{2+} rise (mean ± SEM [nM]) of all cells studied in each area was as follows: hypothalamus (66 ±

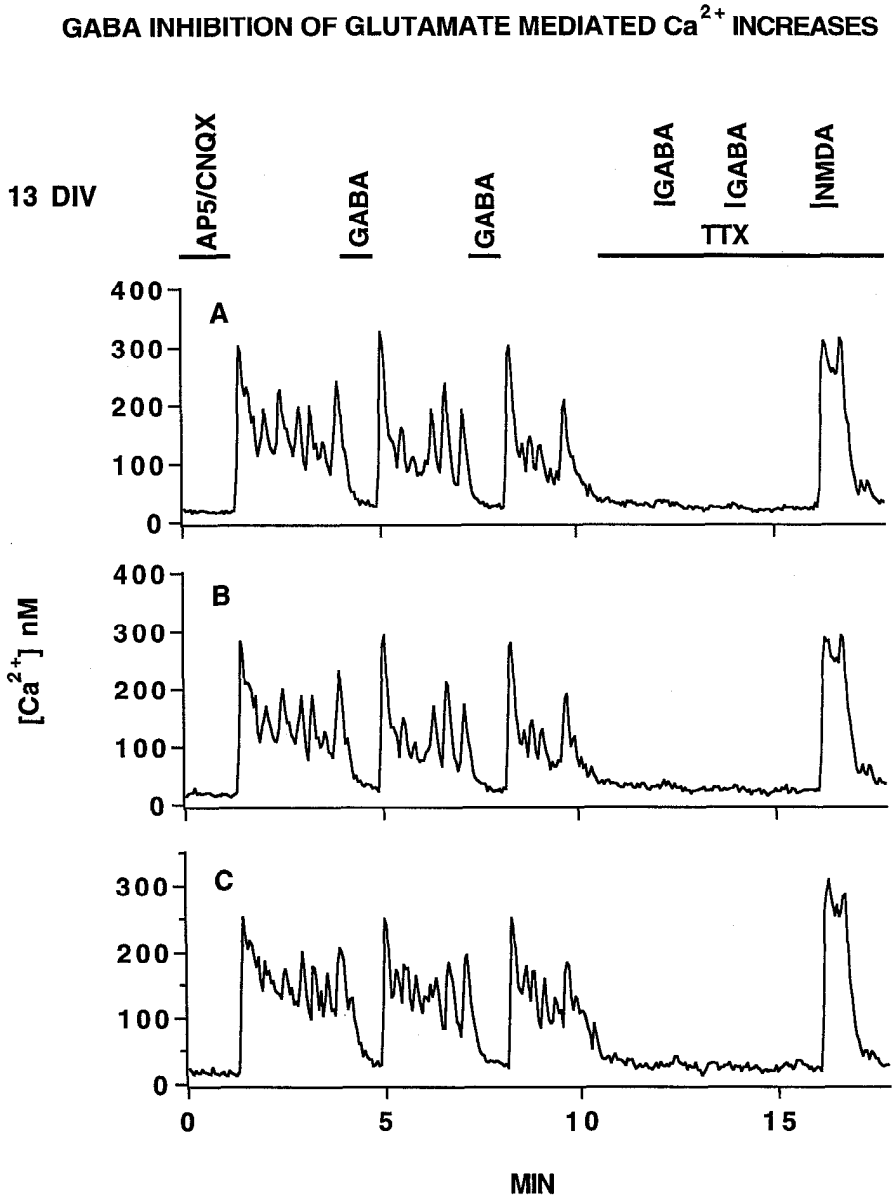

Figure 6. Spontaneous activity in E18 neurons was completely suppressed by the addition of 20 μM GABA. By 13 DIV, 20 μM GABA was unable to induce Ca^{2+} rises in the majority of neurons. 1 μM TTX was used to suppress endogenous synaptic activity.

9), cortex (151 ± 13), hippocampus (93 ± 10), thalamus (51 ± 7), medulla (80 ± 8), colliculus (79 ± 8), striatum (69 ± 8), and olfactory bulb (41 ± 5). When neurons (n = 739) from the hippocampus, cortex, hypothalamus, spinal cord, striatum, and olfactory bulb were studied after 3 weeks in culture, the GABA antagonist bicuculline (20 μM) elicited either no change or an increase in intracellular Ca^{2+}. Less than 1% of the 3 week old cells showed a bicuculline-induced Ca^{2+} decrease. These data indicate that GABA had reversed its role and become a Ca^{2+}-depressing transmitter in these more mature neurons, consistent with our previously described experiments focusing on hypothalamic neurons.

Discussion

Mechanism of GABA-induced Ca^{2+} rise

In young hypothalamic neurons GABA increased Ca^{2+} even during blockade of action potentials with TTX. This indicates that the Ca^{2+} rise was not due to secondary release of some other transmitter initiated by GABA. The effect was completely blocked by bicuculline, indicating the specific action of the $GABA_A$ receptor; the role of the $GABA_A$ receptor was further supported by the activity of muscimol, a $GABA_A$ receptor agonist that also produced a Ca^{2+} rise in young neurons.

The blockade of the Ca^{2+} rise by cadmium indicates that the Ca^{2+} entered the cell from the extracellular milieu. The blockade of most of the Ca^{2+} rise by nimodipine indicated the activation of L-type voltage-gated Ca^{2+} channels to be the primary mechanism of Ca^{2+} entry into the cell. Together these data suggest that GABA opens Cl^- channels that depolarize the neuron; the depolarization activates voltage gated Ca^{2+} channels that allow the entry of extracellular Ca^{2+}. This phenomenon has apparently not been studied previously in hypothalamic neurons, but work in other parts of the nervous system including the retina, hippocampus and spinal cord suggest a parallel function for GABA in early development. In studies of the developing avian retina, spinal cord, rat neocortex, and neonatal pituitary cells *in vitro*, activation of the $GABA_A$ receptor has been reported to elicit a depolarizing current that in turn activates voltage dependent

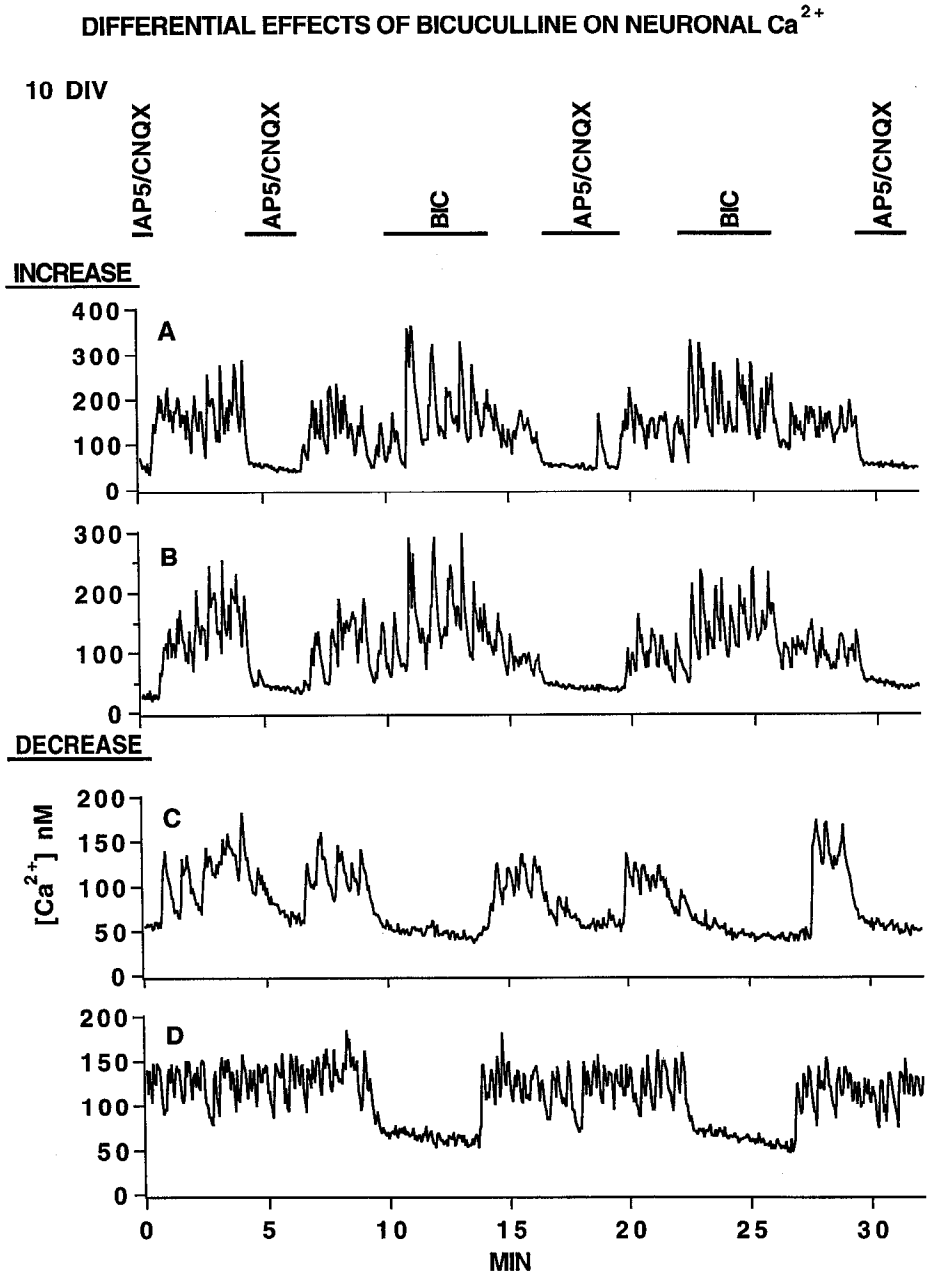

Figure 7. The reversal of the response to bicuculline was most strikingly seen at 10 DIV. Bicuculline reproducibly potentiates Ca^{2+} levels in *A* and *B*, whereas Ca^{2+} levels in *C* and *D* were suppressed. Complex synchronized Ca^{2+} transients are seen in neurons *A* and *B* in an unblocked state, both in the presence and absence of bicuculline. To verify that the cells were neurons, 100 μM NMDA was used to stimulate a Ca^{2+} rise (not shown).

Ca^{2+} channels (Yuste and Katz, 1991; Horvath et al., 1993; Yamashita and Fukuda, 1993). Similarly, in a cell line developed from LHRH secreting neurons, GABA elicited a Ca^{2+} rise, and depending on the chloride equilibrium potential, induced action potentials (Hales et al., 1994). Application of GABA in early but not late embryogenesis elicited action potentials in the developing chick spinal cord (Obata et al., 1978). Embryonic hippocampal cells showed depolarizing responses to nanomolar concentrations of GABA agonists (Fiszman et al., 1990). Hippocampal cells showed giant depolarizing potentials during the first week of postnatal development that appeared to be due to GABA release; these disappeared by P12 (Ben-Ari et al., 1989). Additional mechanisms of GABA-induced Ca^{2+} rises may exist in other cells. For instance, GABA stimulation of cerebellar granule cells evoked a Ca^{2+} rise that took 2 min to develop, and did not recover even 15 min after washout of the GABA (Connor et al, 1987). In contrast, hypothalamic neurons in the present study showed an immediate (2–4 sec) Ca^{2+} rise in response to GABA, and a rapid (seconds) recovery upon GABA washout.

A depolarizing response to GABA can be found in restricted regions of adult hippocampal neurons. GABA applied to dendrites caused a depolarization; in contrast, application to the perikaryon elicited a hyperpolarizing response (Anderson et al., 1980; Alger and Nicoll, 1982). Axons of developing retinal ganglion cells were depolarized by GABA agonists; this sensitivity was lost or reduced in the adult optic nerve (Sakatani et al., 1992; Lim et al., 1993). An extreme example of depolarization through the opening of Cl^- channels is found outside the brain, and even outside the animal kingdom. Algae cells that normally have a negative membrane potential show a slow action potential that, rather than based on Na^+ influx, is based on efflux of high levels of internal Cl^- (Gaffey and Mullins, 1958; Kishimoto, 1965).

In parallel experiments, glycine also evoked a modest Ca^{2+}

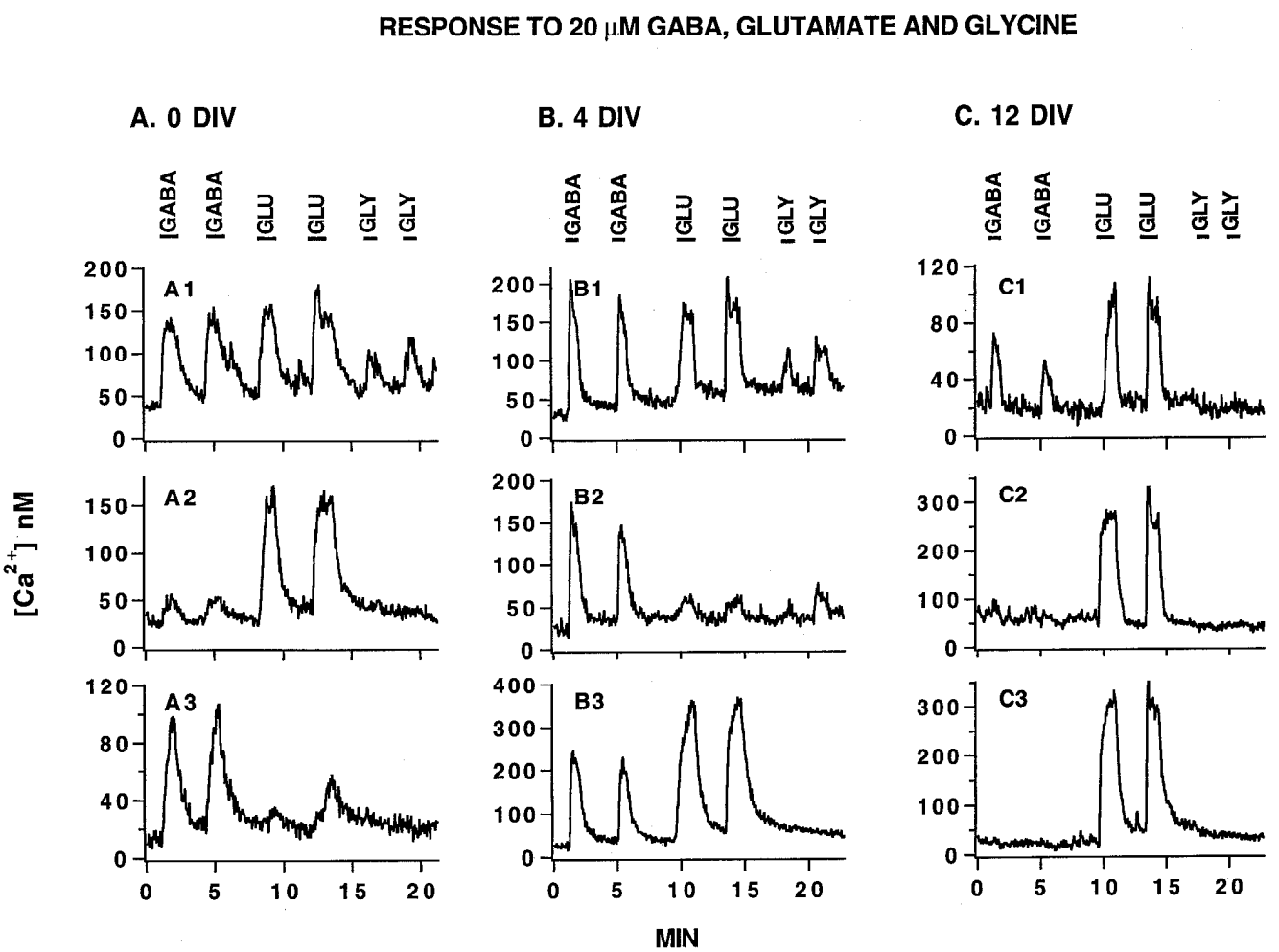

Figure 8. Representative cells plated at E15 are shown at three time points (0, 4, 12 DIV) during development. Cells were treated to the repeated application of 20 μM GABA, 20 μM glutamate, and 20 μM glycine. Of note is the heterogeneous, yet reproducible response to the application of the neurotransmitter receptor agonists. Perfusion solutions contained 1 μM TTX.

rise in our developing hypothalamic neurons, probably due to opening Cl^- channels by strychnine-sensitive glycine receptors, with subsequent activation of voltage regulated Ca^{2+} channels. The ability of glycine to evoke a Ca^{2+} rise was lost in older hypothalamic neurons. Strychnine-sensitive glycine receptor activation evoked depolarizations in hippocampal neurons from rats less than 4 d old (Ito and Cherubini, 1991) and in developing spinal cord motoneurons (Wu et al., 1992).

In the spinal cord, all cells that showed a Ca^{2+} rise in response to GABA also showed one to glycine, and the magnitude of their rise was high correlated (Reichling et al., 1994). In contrast, not all hypothalamic cells that responded to GABA also responded to glycine, although all cells that did respond to glycine also responded to GABA. In cells that did respond to both, in some the response was larger to GABA, and in others the response was larger to glycine. This difference between the hypothalamus and spinal cord Ca^{2+} response may be due to the absence of glycine receptors from some hypothalamic neurons (van den Pol and Gorcs, 1988). Other neurons that do express the glycine receptor in the hypothalamus may have more glycine than GABA receptors. All cells we have examined with ultrastructural immunocytochemistry in the adult hypothalamus were postsynaptic to GABA immunoreactive boutons (Decavel and van den Pol, 1990) and all neurons tested with patch clamp recording show GABA responses (Randle et al., 1986; van den Pol et al., 1995). These data suggest that all hypothalamic neurons probably have GABA receptors, further supporting the importance of early GABA neurotransmission.

Developmental changes in GABA-induced Ca^{2+} response

In early hypothalamic development, GABA increases Ca^{2+}, and in more mature neurons, GABA either has no effect on Ca^{2+} or decreases it. What is the substrate underlying this change? One possibility is that the membrane potential changes with development. Changes in membrane potential would lead to differences in Cl^- movement.

It is unlikely the developmental changes in the GABA-induced Ca^{2+} response are due to changes in extracellular Cl^- because in our experiments the external Cl^- levels were held at a constant level. Changes in intracellular Cl^-, on the other hand, may play an important role in the developmental alteration in GABA activity by changing the equilibrium potential of Cl^-, thereby influencing the voltage change induced by GABA elicited opening of Cl^- channels. One cellular mechanism for this may be through developmental changes in the operation of Cl^- transporters. Different cells may have different Cl^- transporter mechanisms. For instance, hippocampal granule cells can be depolarized by GABA, while at the same stage of development,

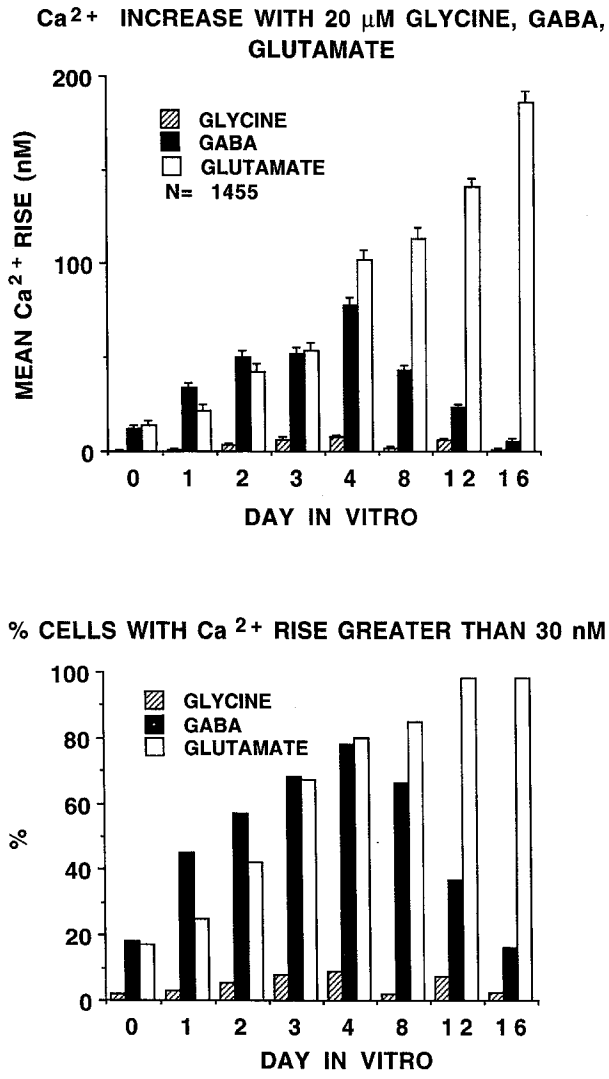

Figure 9. *A*, The mean Ca^{2+} rise to the application of 20 μM glycine, glutamate and GABA is plotted over time in culture (0–16 DIV). The mean response to GABA peaks at 4 DIV, whereas the response to glutamate continues to rise. Bars represent SEM. *B*, At each time point the total number of cells that responded with a minimum rise of 30 nM to the application of agonist is plotted. A greater percentage of cells responded to the addition of GABA than glutamate over the first 3 d in culture. All data were from the same initial plating of cells.

pyramidal cells are hyperpolarized; this differential effect has been attributed to Cl^- transporters of different polarities and is dependent on the different reversal and resting membrane potentials (RMP) of the two cell types (Misgeld et al., 1986). Reduced outward transport of Cl^- was reported in younger hippocampal neurons (Luhmann and Prince, 1991), indicating that the full activity of outward Cl^- transport may develop late. The reversal potential for IPSPs changes during development relative to the RMP. For instance in hippocampal CA1 neurons, the Cl^- dependent IPSP reversal potential in neurons 2–5 d after birth was near the RMP, but by 15–20 d after birth the reversal potential was 25 mV more negative than the RMP (Zhang et al., 1991).

Intracellular changes in Cl^- appear the most likely ionic mechanism to explain the shift from Ca^{2+} elevating to depressing. However, bicarbonate may also pass through Cl^- channels, and may play a role in GABA responses (Kaila et al., 1993). Opening the Cl^- channel could result in HCO_3^- passing out of the cell, depolarizing the cell. An electrophysiological study in the hippocampus argued that HCO_3^- appeared unlikely to play a significant role in depolarizing GABA responses in hippocampal pyramidal cells (Grover et al., 1993).

The effect of GABA in reducing intracellular Ca^{2+} is particularly dramatic in older neurons that show an elevated Ca^{2+} due to glutamate excitation. GABA acting as an inhibitory transmitter reduces the glutamate-mediated activity either by hyperpolarization or by a slight depolarization and current shunt, resulting in a large decrease in Ca^{2+}. Parallel decreases in Ca^{2+} can be produced by blocking action potentials with TTX, or blocking glutamate receptors with AP5/CNQX.

Changes in the molecular expression of $GABA_A$ receptor subunits occur during development (Fritschy et al., 1994; Mathews et al., 1994). Physiologically, GABA receptors show less desensitization in young neurons than in adult cells (Oh and Dichter, 1992). A change in the density of GABA receptors (Hansen et al., 1987, 1991) or in the subunit configuration (Fritschy et al., 1994; Mathews et al., 1994) could alter the Cl^- movement through the channel.

Developmental role of GABA-induced Ca^{2+} rises

In early hypothalamic development GABA appears to play a greater role than the excitatory transmitter glutamate in evoking Ca^{2+} rises. This is consistent with experiments near the beginning of hypothalamic neurogenesis (E15) when all cells tested with patch-clamp recording showed a large response to GABA, but only some showed a response to glutamate (van den Pol et al., 1995). Studies in the spinal cord showed that GABA receptors are expressed in early embryogenesis and may be found even earlier than glutamate receptors (Walton et al., 1993). GABA and its synthetic enzyme are found in E6 neurons in the chick retina (De Mello et al., 1976; Frederick, 1987); this is prior to significant synaptogenesis described at E14 (Sheffield and Fischman, 1970). GABA may also be released during development, perhaps even before synaptogenesis (Gordon-Weeks et al., 1984; Balcar et al., 1986; Hicks et al., 1986); GABA release could be induced from a cell fraction containing axon endings prior to the time when the synaptic proteins P65 and synaptophysin could be detected immunocytochemically (Taylor et al., 1990). Spontaneous hyperpolarizing potentials associated with GABA transmission have been found developmentally later than depolarizing potentials attributed to glutamate in the retina (Rorig and Grantyn, 1993). NMDA receptor currents were found in the somatosensory cortex before inhibitory currents (Agmon and O'Dowd, 1992). It is possible that some of the depolarizing potentials throughout the brain in early stages of development could be elicited by spontaneous GABA release.

A number of studies have shown that intracellular Ca^{2+} may play important roles in neurite outgrowth *in vitro* (Mattson and Kater, 1987). GABA can stimulate process outgrowth; hippocampal neurons raised in the presence of GABA receptor antagonists had fewer neurites and a smaller total neuritic length than controls (Barbin et al., 1993). GABA also influenced neuritic branching and synaptogenesis in avian retina and brain cultures (Spoerri, 1988; Michler, 1990). GABA agonists have been reported to alter the expression of GABA receptors and to influence the ultrastructure of developing cerebellar granule cells (Hansen et al., 1987, 1988, 1991; Belhage et al., 1988).

Another role for GABA, in collaboration with glutamate, may be to regulate and stabilize the Ca^{2+} setpoint of developing neu-

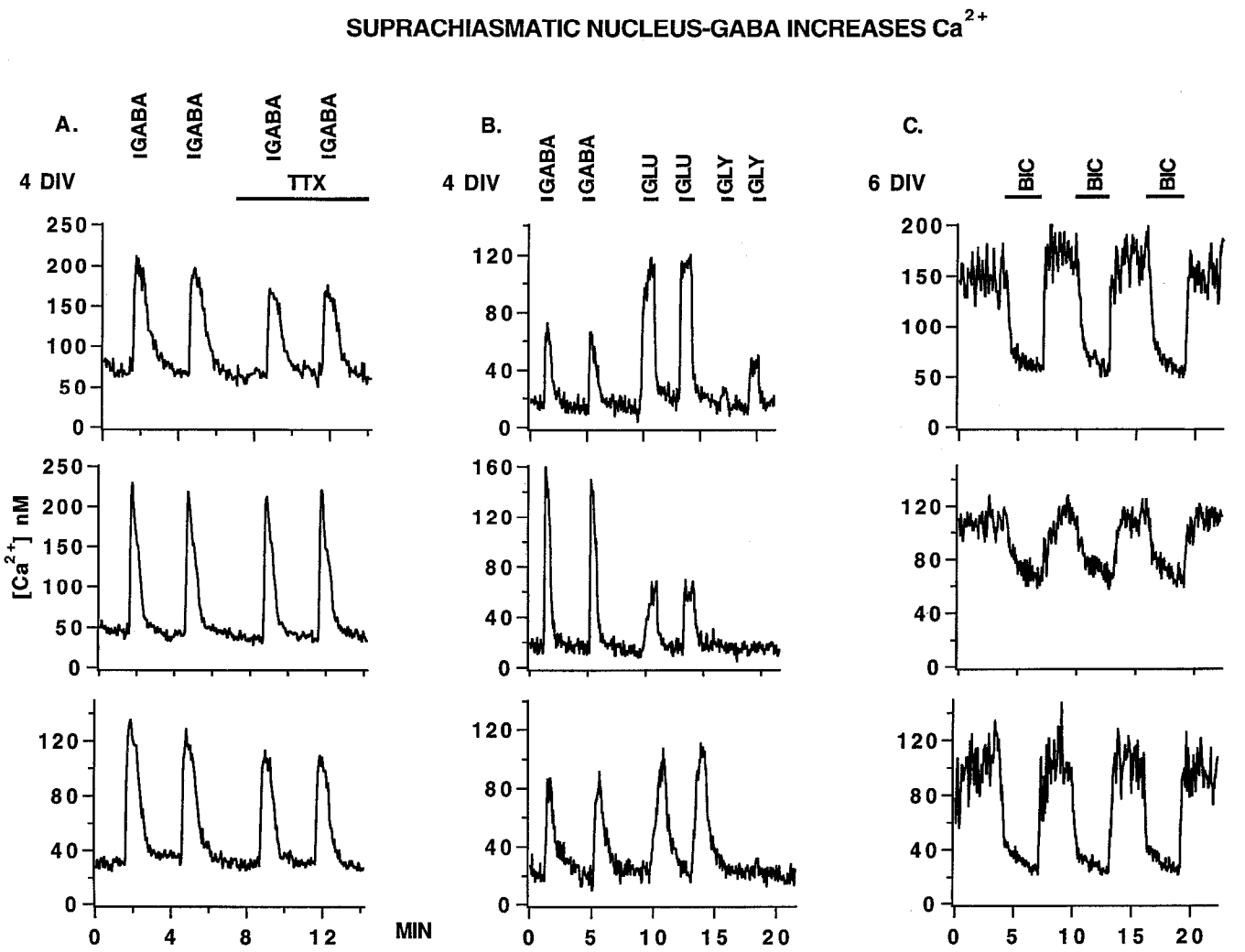

Figure 10. Characteristic responses of neurons from the suprachiasmatic region of the hypothalamus are shown. *A*, Both in the absence and presence of TTX (1 μM), Ca^{2+} levels increased in response to 20 μM GABA. 4 DIV. *B*, Equimolar concentrations of GABA, glutamate, and glycine (20 μM) evoked reproducible increases in Ca^{2+}. 4 DIV. *C*, The addition of 20 μM bicuculline depressed basal Ca^{2+} levels in neurons constantly perfused with AP5 (100 μM) and CNQX (10 μM) after 6 DIV, indicating that GABA was being released by cells in these cultures.

rons. In neurons *in vitro* for 4 d, examined in the present study, the addition of either GABA or glutamate evoked an increase in Ca^{2+}. However, the combined addition of GABA and glutamate resulted in a Ca^{2+} rise of an intermediate value between the two, regardless of which amino acid initially produced the greater Ca^{2+} rise. In neurons in which GABA induced a greater Ca^{2+} rise than glutamate did, the addition of glutamate to GABA caused a decrease in Ca^{2+}, suggesting a paradoxical reversed Ca^{2+} inhibitory role for glutamate in some developing hypothalamic neurons. The intermediate, rather than additive rise in Ca^{2+} in the presence of both Ca^{2+}-stimulating GABA and glutamate suggests that the combined effect of both would not lead to Ca^{2+} excitotoxicity, but rather would tend to stabilize with a more modest Ca^{2+} rise. Many cellular processes function optimally within a narrow range of cytoplasmic Ca^{2+} concentrations. Neurons may die both as a result of Ca^{2+} levels that are too high, for instance caused by glutamate excitotoxicity (Olney and Sharpe, 1969; Choi, 1988), but neurons may die also if Ca^{2+} levels are too low (Franklin and Johnson, 1992).

In older neurons from the suprachiasmatic nucleus, GABA can inhibit ultradian Ca^{2+} oscillations (van den Pol et al., 1992). GABA release from immature neurons may serve to synchronize cellular events via the increase of intracellular Ca^{2+}. Experiments with 2-deoxyglucose suggest that cells in the SCN *in vivo* (Reppert and Schwartz, 1984) or *in vitro* (Shibata and Moore, 1988) show an orchestrated rhythm of uptake in early stages of development. A potential explanation for this, based on the data in the present study, may be that GABA released by young SCN cells could serve to orchestrate the juvenile neurons in the SCN that are involved in circadian timekeeping.

The studies described here focus on hypothalamic neurons. Our experiments surveying seven other regions of the brain indicated that GABA elicited a Ca^{2+} rise in the majority of all developing neurons tested. Together with work from other labs described above, our data support the probability that the early role of GABA as an excitatory transmitter is not restricted to a few brain regions, but rather is widespread throughout the CNS.

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