Neuron, Vol. 15, 1287–1298, December, 1995, Copyright © 1995 by Cell Press

GABA and Glutamate Depolarize Cortical Progenitor Cells and Inhibit DNA Synthesis

Joseph J. LoTurco,* David F. Owens,† Mark J. S. Heath,‡ Marion B. E. Davis,§ and Arnold R. Kriegstein§
*Department of Physiology and Neurobiology
University of Connecticut at Storrs
Storrs, Connecticut 06269
†Center for Neurobiology and Behavior
‡Department of Anesthesiology
§Department of Neurology
College of Physicians and Surgeons
Columbia University
New York, New York 10032

Summary

We have found that, during the early stages of cortical neurogenesis, both GABA and glutamate depolarize cells in the ventricular zone of rat embryonic neocortex. In the ventricular zone, glutamate acts on AMPA/kainate receptors, while GABA acts on $GABA_A$ receptors. GABA induces an inward current at resting membrane potentials, presumably owing to a high intracellular Cl^- concentration maintained by furosemide-sensitive Cl^- transport. GABA and glutamate also produce increases in intracellular Ca^{2+} in ventricular zone cells, in part through activation of voltage-gated Ca^{2+} channels. Furthermore, GABA and glutamate decrease the number of embryonic cortical cells synthesizing DNA. Depolarization with K^+ similarly decreases DNA synthesis, suggesting that the neurotransmitters act via membrane depolarization. Applied alone, $GABA_A$ and AMPA/kainate receptor antagonists increase DNA synthesis, indicating that endogenously released amino acids influence neocortical progenitors in the cell cycle. These results demonstrate a novel role for amino acid neurotransmitters in regulating neocortical neurogenesis.

Introduction

The cells of the cerebral cortex, both neurons and glia, originate from the ventricular zone (VZ) and subventricular zone (SVZ) of the developing telencephalon (Boulder Committee, 1970). It is becoming increasingly clear that important developmental decisions are made by proliferating cells within these zones. Cortical neurons become committed to a laminar fate through interactions with their environment (McConnell, 1988; McConnell and Kaznowski, 1991); similarly, pyramidal or nonpyramidal cell fate may be determined by local cues within the VZ (Luskin et al., 1993; Mione et al., 1994). In addition, imaging and lineage experiments indicate that cortical progenitors migrate within the VZ (Fishell et al., 1993; Walsh and Cepko, 1993). An understanding of cellular development in the cortex will therefore require an analysis of signals that influence neocortical progenitors in the proliferative zones.

In the embryonic rat cortex, gap junction channels couple VZ cells into adjacent, radially arrayed clusters (LoTurco and Kriegstein, 1991). The number of cells coupled in a cluster decreases from ~60 at embryonic day 15 (E15) to ~6 at E19. Columns of coupled cells are therefore present within the proliferative zone throughout the most intense periods of neurogenesis and undergo a gradual reduction in size. The coupled cell clusters define functional units within the proliferative zone of embryonic cortex, and local environmental factors could influence cells within a cluster by acting through specific membrane receptors on 1 or more cells.

Candidate molecules that may signal neocortical progenitors include growth factors and neurotransmitters. Many neurotransmitters have been localized within or near the VZ and SVZ (Marin-Padilla, 1971; Rickmann et al., 1977; Schlumpf et al., 1980; Wallace and Lauder, 1983; Lauder et al., 1986; Chun et al., 1987; Van Eden et al., 1989; Schwartz and Meinecke, 1992), and it has been hypothesized that these neurotransmitters play a role in cortical development. Previous studies using cultured neurons have shown that the principal excitatory and inhibitory amino acid transmitters in adult cortex, γ-aminobutyric acid (GABA) and glutamate, can regulate the outgrowth of neurites (Hansen et al., 1984; Redburn and Schousboe, 1987; Mattson et al., 1988; Brewer and Cotman, 1989; Lipton and Kater, 1989), influence neuronal survivial (Balazs et al., 1988; Meier et al., 1991; Mount et al., 1993), and, in the case of GABA, serve as a chemoattractant for migrating neurons (Hansen et al., 1987; Behar et al., 1994). To determine how GABA and glutamate may influence the development of cortical cells at earlier proliferative stages, we used in situ whole-cell and perforated-patch clamp methods and Ca^{2+} imaging techniques to record from embryonic neocortical cells in the VZ. We then explored the effects of GABA and glutamate on the mitotic activity of proliferating cortical cells by treating explants with selective agonists and antagonists to the amino acid receptors present on these cells. We found that activation of $GABA_A$ and glutamate AMPA/kainate receptors negatively regulates DNA synthesis in embryonic cortex, most likely through a depolarization-based mechanism.

Results

We have previously shown that clusters of rat embryonic VZ cells are coupled by gap junction channels (LoTurco and Kriegstein, 1991). Each single-cell recording thus measures currents from multiple cells that are electrically coupled (Figure 1). The VZ is a proliferative neuroepithelium and includes cells in all phases of the cell cycle. To test whether coupled clusters include cells synthesizing DNA, we used two labeling techniques. We recorded from rat VZ cells at E17 with biocytin-containing electrodes to stain cell clusters and then incubated explants in [^{3}H]thymidine for 1 hr to label cells in S phase of the cell cycle. After processing, cells were labeled with both biocytin and

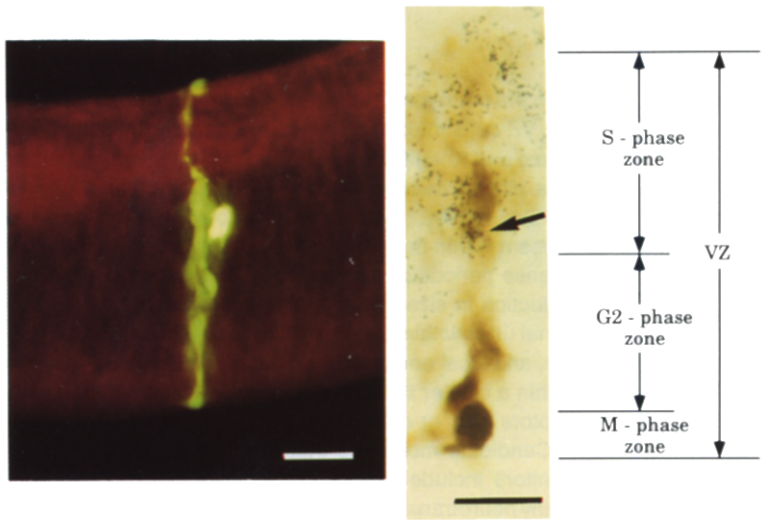

Figure 1. Recorded Cells Are Members of Coupled Cell Clusters in the VZ and Are in the Proliferative Cell Cycle

(Left) A cluster of coupled VZ cells is shown here following intracellular injection of Lucifer yellow at E16.

(Right) A group of coupled cells at E17 was filled with biocytin and then pulsed for 1 hr with [^{3}H]thymidine. Following biocytin processing and autoradiography, a cluster of dye-filled cells can be seen, including some double labeled with both biocytin and [^{3}H]thymidine (arrow), indicating that cells in S phase are members of coupled cell clusters. The silver grains and dye-filled cells are slightly out of focus, as they are in different focal planes. Bars, 25 μm.

[^{3}H]thymidine, indicating that cells in S phase are coupled to other cells in the VZ (Figure 1). In addition, rounded cells at the ventricular surface that appear to be in M phase of the cell cycle were also labeled with biocytin. To confirm that cells in later stages of the cell cycle are also coupled to other VZ cells, we incubated explants in [^{3}H]thymidine for 1 hr and ~8 hr later intracellularly filled cells in the VZ with biocytin. Double-labeled profiles were observed in these experiments as well, indicating that cells in the later G2 phase of the cell cycle are coupled to cells in S phase.

To assay the effects of various neurotransmitters on VZ cells, we used in situ whole-cell patch-clamp recordings on slices and tissue slabs of embryonic rat cortex (Blanton et al., 1989). Our ability to voltage clamp the membrane of a single cell is limited by the large area of membrane composing a coupled cell cluster. On the other hand, we are more likely to measure responses, even in the case of low receptor density, because we have electrical access to a large area of membrane. At E13 and E14, cells in the VZ of rat cortex did not respond to bath application of either glutamate (100 μM) or GABA (30 μM) (n = 12). By E15, however, 42% and 35% of cells (n = 38) depolarized upon the application of GABA and glutamate, respectively. On E16, 100% of cells responded to both amino acids (n = 28). GABA (30 μM) depolarized E16 cells from a resting potential of −60 ± 4 mV to −25 ± 8 mV, and glutamate (300 μM) depolarized E16 cells to −20 ± 6 mV (Figures 2A and 2B). Likewise, both GABA and glutamate induced inward currents in voltage-clamped VZ cells (Figures 2C and 2D). The dose–response characteristics indicate an approximate half-maximal response concentration of 5 μM for GABA and 75 μM for glutamate (Figures 2E and 2F). Several other receptor agonists—glycine, N-methyl-D-aspartate (NMDA), and carbachol—never elicited responses in VZ cells.

The response of embryonic VZ cells to glutamate (Figure 3A) is mediated by non-NMDA glutamate receptors, as bath application of NMDA in the presence of glycine (3 μM) elicited no detectable currents in VZ cells either at depolarized membrane potentials (Figure 3E) or in solutions containing low (0.2 mM) Mg^{2+} concentrations (LoTurco et al., 1991). Kainate (n = 12; Figure 3C) and AMPA (n = 9; Figure 3D) elicited inward currents comparable to those elicited by glutamate. The AMPA/kainate receptor antagonist 6-cyano-7-dinitroquinoxaline-2,3-dione (CNQX; 10 μM; Honore et al., 1988) blocked the current induced by either glutamate or kainate (n = 4; Figure 3B).

We have previously shown that the GABA-induced current in cortical progenitor cells can be antagonized by bicuculline methiodide (BMI) and potentiated by the benzodiazepine diazepam (LoTurco and Kriegstein, 1991), indicating mediation by $GABA_A$ receptors. Confirming that $GABA_A$ receptors are responsible for the GABA response, focal application of the $GABA_A$ agonist muscimol (n = 14) also induced a large current in these cells (Figure 4B), and the $GABA_A$ receptor antagonists BMI (n = 15) and picrotoxin (n = 12) antagonized the response to GABA (Figures 4C and 4D). The $GABA_B$ receptor agonist baclofen (n = 9) produced no obvious current in VZ cells (Figure 4E). This pharmacological profile confirms that $GABA_A$ receptors are present on embryonic VZ cells and account for the GABA responses.

GABA is an inhibitory neurotransmitter in adult cortical neurons, yet application of GABA to VZ cells always resulted in membrane depolarization or an inward current. Because $GABA_A$ channels are permeable to Cl^-, the direction of current flow in response to GABA will depend on the Cl^- gradient across the membrane. With whole-cell patch-clamp recordings, the Cl^- concentration inside the electrode will influence the intracellular Cl^- concentration ($[Cl^-]_i$); however, with a large syncytium of cells, the cytoplasm may not reach full equilibrium with the pipette solution, therefore making estimation of the $[Cl^-]_i$ difficult. To avoid this complication, we used a gramicidin perforated-patch recording method (Abe et al., 1994; Kyrozis and Reichling, 1995). The membrane pores formed by gramicidin are exclusively permeable to monovalent cations and small, uncharged molecules, thus allowing for recordings that leave the $[Cl^-]_i$ undisturbed (Hladky and Haydon, 1972; Myers and Haydon, 1972). Perforated-patch re-

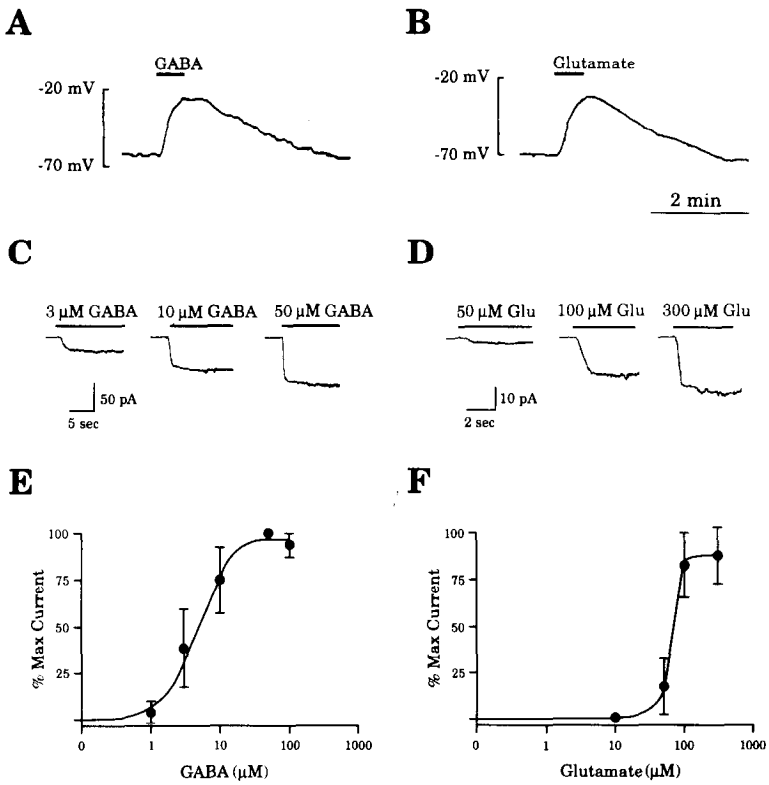

Figure 2. VZ Cells Have Functional GABA and Glutamate Receptors

(A and B) Depolarizing voltage changes were recorded from E16 VZ cells in response to bath application of GABA (30 μM) and glutamate (300 μM). The pipette solution contained 120 mM Cs-gluconate, 10 mM CsCl, 10 mM HEPES (pH 7.3), 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM EGTA. Bar, 2 min.

(C and D) Examples of inward currents induced in individual E17 VZ cells by focal application of increasing concentrations of GABA and glutamate. The pipette solution contained 100 mM CsCl, 30 mM Cs-gluconate, 10 mM HEPES (pH 7.3), 2 mM $CaCl_2$, and 10 mM EGTA.

(E and F) The dose-response characteristics of inward currents obtained by averaging responses of cells to glutamate (n = 6) and GABA (n = 7). The concentration of GABA that produced a half-maximal response was ~5 μM, while the half-maximal dose of glutamate was ~75 μM. All data are from E17 VZ recordings.

cordings were obtained from E16 VZ cells, and GABA application always resulted in either membrane depolarization or an inward current (n = 6). Figure 5A shows the depolarization resulting from the focal application of 30 μM GABA while recording with a perforated-patch electrode in current-clamp mode (left trace) and the inward current induced in the same cell during a similar application of GABA while recording in voltage-clamp mode (right trace).

Since $GABA_A$ channels conduct Cl^-, the depolarization in response to GABA could be explained by a high $[Cl^-]_i$ maintained within VZ cells. Dialyzing cells with a low Cl^-–containing solution during whole-cell recording shifted the relative GABA current reversal potential to more negative values. This suggests that the depolarizing response to GABA is at least in part the result of a Cl^- current and indicates that VZ cells actively maintain a high $[Cl^-]_i$. To test this further, we treated cells with furosemide, a Cl^- transport blocker, and then measured the relative reversal potential of GABA currents. Furosemide shifted the reversal potential from approximately −5 mV to approximately −45 mV (Figures 5B and 5C), consistent with the hypothesis that VZ cells accumulate intracellular Cl^- and thus have a depolarizing response to GABA.

A cytosolic event likely to be triggered by amino acid–induced depolarization of VZ cells is an increase in $[Ca^{2+}]_i$. To test whether GABA and glutamate trigger an increase in $[Ca^{2+}]_i$ in VZ cells, we first used acute slices of embryonic cortex and the Ca^{2+} indicator Fluo 3-AM. This method permitted visualization of cells within the VZ. Cortical slices were obtained at E16, an age at which the VZ is relatively large, and Fluo 3–loaded cells were imaged using laser confocal microscopy (Figure 6A). Applications of GABA (30 μM) and glutamate (50 μM) produced reversible increases in $[Ca^{2+}]_i$ in cells throughout the VZ (Figure 6A). Depolarizing cells by application of KCl (60 mM) produced similar increases of $[Ca^{2+}]_i$, consistent with the presence of voltage-gated Ca^{2+} channels (VGCCs) in these cells.

Because the Ca^{2+} changes observed in cells in situ could be produced indirectly by stimulation of cells outside the VZ, a tissue printing method was employed to obtain acutely isolated VZ cells (Figure 6B), which were then loaded with the Ca^{2+} indicator dye Fura 2-AM. This technique also allowed a more detailed quantitative analysis of the effects of amino acid transmitters on VZ cells. Bath application of GABA (30 μM) produced an increase in $[Ca^{2+}]_i$ of 39 ± 19 nM (mean ± SD; n = 22), and glutamate (50 μM) induced an increase in $[Ca^{2+}]_i$ of 38 ± 19 nM (n = 23; Figure 6C). Depolarization by bath application of KCl (60 mM) induced an increase in $[Ca^{2+}]_i$ of 32 ± 16 nM (n = 18), consistent with the presence of VGCCs in VZ cells. To test whether the GABA- and glutamate-induced increase in $[Ca^{2+}]_i$ was mediated by depolarization-induced activation of VGCCs, La^{3+} was used to block VGCCs (Reichling and MacDermott, 1991). La^{3+} (10 μM) entirely abolished the increase in $[Ca^{2+}]_i$ produced by KCl (Figure 6C). Likewise, the GABA-induced $[Ca^{2+}]_i$ increase was entirely blocked by La^{3+}, indicating that VGCCs mediate the GABA response. La^{3+} reduced the glutamate-induced $[Ca^{2+}]_i$ increase to 18 ± 10 nM (mean ± SD; n = 18), suggesting that VGCCs are at least partly involved in the glutamate-induced response.

The hypothesis that activation of glutamate and GABA

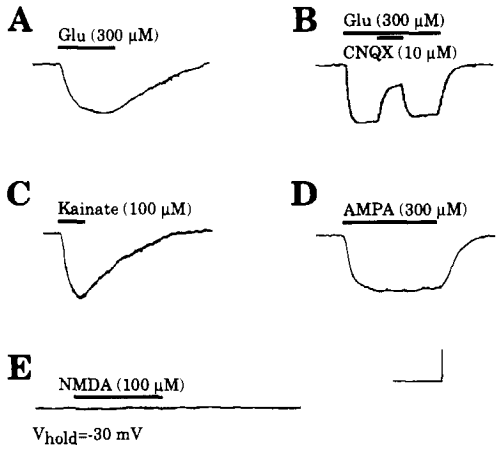

Figure 3. The Glutamate Receptor on VZ Cells Is an AMPA/Kainate-Sensitive Receptor

(A) Glutamate (300 μM) elicits an inward current in VZ cells. Drug was bath applied, and the recording pipette contained 120 mM Cs-gluconate, 10 mM CsCl, 10 mM HEPES (pH 7.3), 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM EGTA.

(B) The current induced by glutamate (300 μM) is antagonized by CNQX (10 μM). Drugs were focally applied. The pipette solution contained 100 mM CsCl, 30 mM Cs-gluconate, 10 mM HEPES (pH 7.3), 2 mM $CaCl_2$, and 10 mM EGTA.

(C) Kainate (100 μM) elicits an inward current in VZ cells. Drug was bath applied, and the recording pipette contained 120 mM Cs-gluconate, 10 mM CsCl, 10 mM HEPES (pH 7.3), 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM EGTA.

(D) AMPA (300 μM) elicits an inward current in VZ cells. Drug was focally applied. The pipette solution contained 100 mM CsCl, 30 mM Cs-gluconate, 10 mM HEPES (pH 7.3), 2 mM $CaCl_2$, and 10 mM EGTA.

(E) NMDA (100 μM) induced no detectable currents from a holding potential of −30 mV. Recording was from the same cell as shown in (A). The pipette solution and drug application method were the same as in (A).

Recordings were from E17 VZ cells, except that in (D), which was from an E16 VZ cell. Bars, 10 s and 25 pA (A and C), 5 s and 50 pA (B), 6.9 s and 37.5 pA (D), 15 s and 25 pA (E).

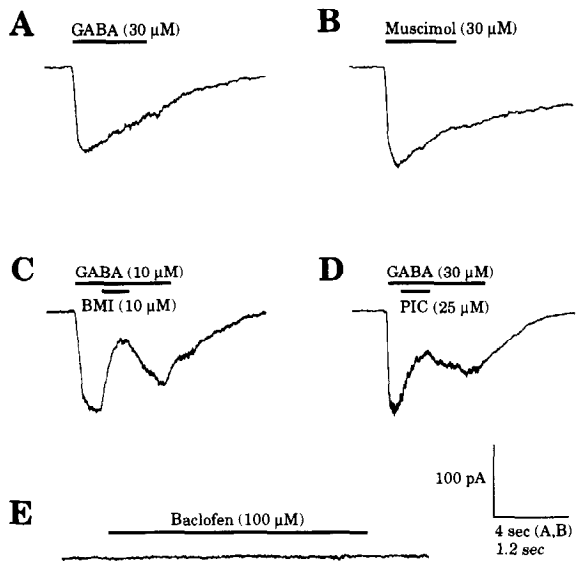

Figure 4. The GABA Receptor on VZ Cells Is a $GABA_A$ Receptor

(A and B) The inward current induced by GABA (30 μM) is mimicked by the $GABA_A$ agonist muscimol (30 μM). Recordings are from the same cell.

(C and D) The GABA-induced current is blocked by the $GABA_A$ antagonists bicuculline methiodide (BMI; 10 μM) and picrotoxin (PIC; 25 μM).

(E) The $GABA_B$ agonist baclofen (100 μM) produced no current in these cells.

All recordings are from E16 VZ cells. The pipette solution contained 100 mM CsCl, 30 mM Cs-gluconate, 10 mM HEPES (pH 7.3), 2 mM $CaCl_2$, and 10 mM EGTA. All drugs were focally applied.

receptors influences the cell cycle of cortical progenitors was tested using explants of embryonic cortex exposed to amino acid receptor agonists and antagonists for 5 hr in the presence of [^{3}H]thymidine. Alterations in S phase of the cell cycle would be reflected by changes in the amount of [^{3}H]thymidine incorporated into DNA of the population of cortical progenitors. Both GABA and kainate caused a significant decrease in the incorporation of [^{3}H]thymidine into E16 and E19 cortical explants, but not in younger E14 explants (Figure 7). The difference between younger and older explants is consistent with the absence of amino acid–induced currents in E14 VZ cells and supports the conclusion that functional amino acid receptors are not present on VZ cells at E14. In addition, the GABA-induced decrease in DNA synthesis was blocked by the GABA antagonist BMI, and, similarly, the kainate-induced decrease was blocked by the AMPA/kainate receptor antagonist CNQX (Figures 7A and 7B). The BMI concentration (50 μM) used in these experiments was found to produce a nearly complete block of inward currents induced by saturating GABA concentrations. Therefore, activation of either $GABA_A$ or kainate receptors reduces DNA synthesis in cortical progenitor cells. In contrast, NMDA did not change [^{3}H]thymidine incorporation (data not shown), indicating that the amino acid–induced decrease is specific for receptors present on cortical progenitors. Furthermore, the effects of kainate and GABA were reversible (Figures 7A and 7B, post-GABA and post-kainate), thereby ruling out the possibility that rapid cell death caused the agonist-induced decrease in DNA synthesis. In addition to [^{3}H]thymidine incorporation, we used bromodeoxyuridine (BrdU) incorporation to determine the number of cells in S phase (mitotic index) during exposure to GABA and glutamate. When E18 explants were treated with glutamate or GABA for 12 hr, the number of cells that incorporated BrdU was approximately half of the number of cells incorporating BrdU in controls (Figure 8A).

While GABA and glutamate receptor channels conduct different ions, they have similar effects on both membrane potential and DNA synthesis. We therefore tested whether depolarization alone could down-regulate DNA synthesis. When explants were bathed in 20 mM KCl, which produced a similar degree of depolarization as saturating GABA and glutamate concentrations, DNA synthesis was decreased (Figure 8B). In addition, we found that the GABA-induced inhibition in DNA synthesis is depolarization dependent. As noted above, GABA is depolarizing in embryonic VZ cells, presumably owing to a high $[Cl^-]_i$ maintained by a furosemide-sensitive Cl^- transport process. When the reversal potential of GABA was shifted negatively by treatment with furosemide, GABA applica-

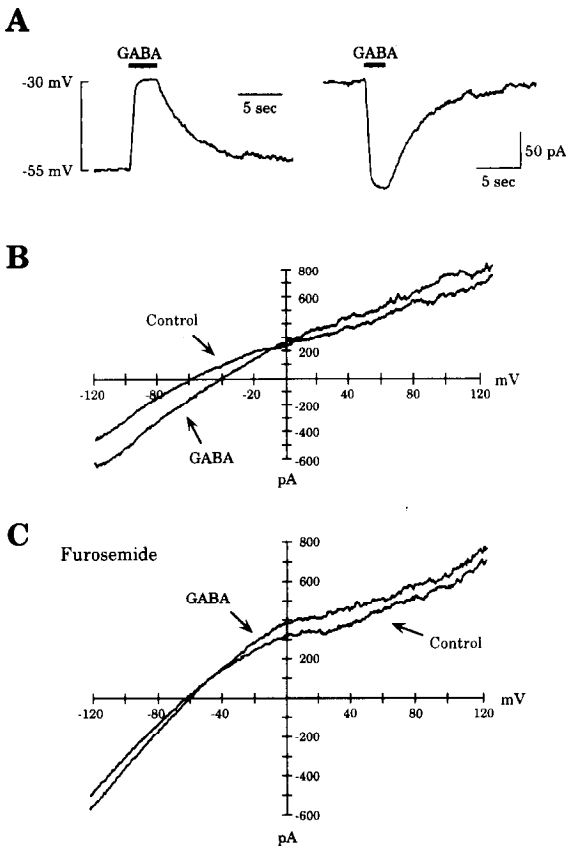

Figure 5. High $[Cl^-]_i$ Contributes to the Depolarizing GABA Response in VZ Cells

(A) GABA (30 μM) induces both membrane depolarization (left) and inward current (right) in gramicidin perforated-patch recordings from E16 VZ cells. Both recordings are from the same cell. The pipette solution contained 100 mM CsCl, 30 mM Cs-gluconate, 10 mM HEPES (pH 7.3), 2 mM $CaCl_2$, 10 mM EGTA, and 1 μg/ml gramicidin.

(B) Current-voltage plot before and after the application of GABA, indicating a reversal potential for the GABA-induced current of approximately −5 mV. The current-voltage relation was determined by making a ramp voltage change from −120 to +120 mV.

(C) The Cl^- transport blocker furosemide (5 mM) shifts the GABA-induced current reversal potential to a more hyperpolarized level. Recordings are from E18–E19 VZ cells. Drugs were bath applied, and the pipette solution contained 120 mM Cs-gluconate, 10 mM CsCl, 10 mM HEPES (pH 7.3), 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM EGTA.

tion was no longer depolarizing. Under these conditions, [^{3}H]thymidine incorporation was not reduced (Figure 8B). Thus, depolarization is both necessary and sufficient for the down-regulation of DNA synthesis.

While the above experiments indicate that exogenously applied amino acid neurotransmitters are capable of reducing DNA synthesis, they do not demonstrate that such regulation occurs during normal cortical development. If endogenous neurotransmitters down-regulate DNA synthesis, then receptor antagonists alone should increase DNA synthesis. Furthermore, since transmitter-containing cells, axons, and growth cones increase throughout early cortical development (Lauder et al., 1986; Parnavelas and Cavanagh, 1988; Van Eden et al., 1989; Blanton and Kriegstein, 1991), antagonists might increase DNA synthesis to a greater extent in older, E19 explants as compared to younger, E16 explants. We found that, when either CNQX (10 μM) or BMI (50 μM) was applied to E16 explants, there was no significant effect on DNA synthesis (data not shown). In contrast, in E19 explants both CNQX and BMI significantly increased [^{3}H]thymidine incorporation (Figures 9A and 9B). Thus, endogenously released amino acid neurotransmitters down-regulate DNA synthesis at a defined stage in cortical development. In addition, [^{3}H]thymidine incorporation was significantly reduced in the 5 hr interval following exposure to receptor antagonists. This rebound reduction could result from an up-regulation in receptor function during the exposure to antagonists or from a reduction in the number of cells available to enter S phase.

Because we record from VZ cells in situ in acute cortical explants, we were also able to test whether endogenous agonists produce tonic currents in VZ cells during cortical development. We observed a small outward shift in current, presumably reflecting the blockade of a tonic inward current, in response to perfusion of the $GABA_A$ antagonist BMI (100 μM) in VZ cells at E17 (n = 3) and E19 (n = 5) (Figure 9C). This observation suggests that $GABA_A$ receptors are tonically activated in these cells during embryonic development. Similarly small outward current shifts in VZ cells in response to the AMPA/kainate receptor antagonist CNQX (100 μM) were also observed, but less frequently.

Discussion

$GABA_A$ and AMPA/Kainate Glutamate Receptor Subtypes in Proliferating Cortical Cells

The present results suggest a previously unrecognized role for amino acid transmitters in corticogenesis. Even before their final mitotic division, cortical progenitor cells express functional receptors for the principal excitatory and inhibitory transmitter substances, GABA and glutamate, used for synaptic communication by adult cortical neurons. Both transmitters depolarize cortical precursor cells before their terminal mitosis and decrease the number of cells in S phase of the cell cycle. In contrast, the NMDA-type glutamate receptor is expressed after mitosis, when migrating neurons reach the cortical plate (LoTurco et al., 1991). This precocious expression of functional amino acid receptors raises the possibility that they play a role in early stages of cortical development, and the temporal order of expression suggests that each receptor may have a distinct function during stages of proliferation, differentiation, and synaptogenesis. This hypothesis is supported by molecular studies demonstrating a changing expression pattern of mRNAs for different subunit combinations of glutamate and $GABA_A$ receptors at different developmental stages (Monyer et al., 1991; Araki et al., 1992; Laurie et al., 1992; Poulter et al., 1992). GABA and glutamate have been shown to have a variety of trophic effects on postmitotic neurons in vitro (Redburn and Schousboe, 1987; Brewer and Cotman, 1989; Lipton and Kater, 1989). Our data demonstrate that GABA and glutamate have effects even earlier in neural development and influence cell cycle events in situ.

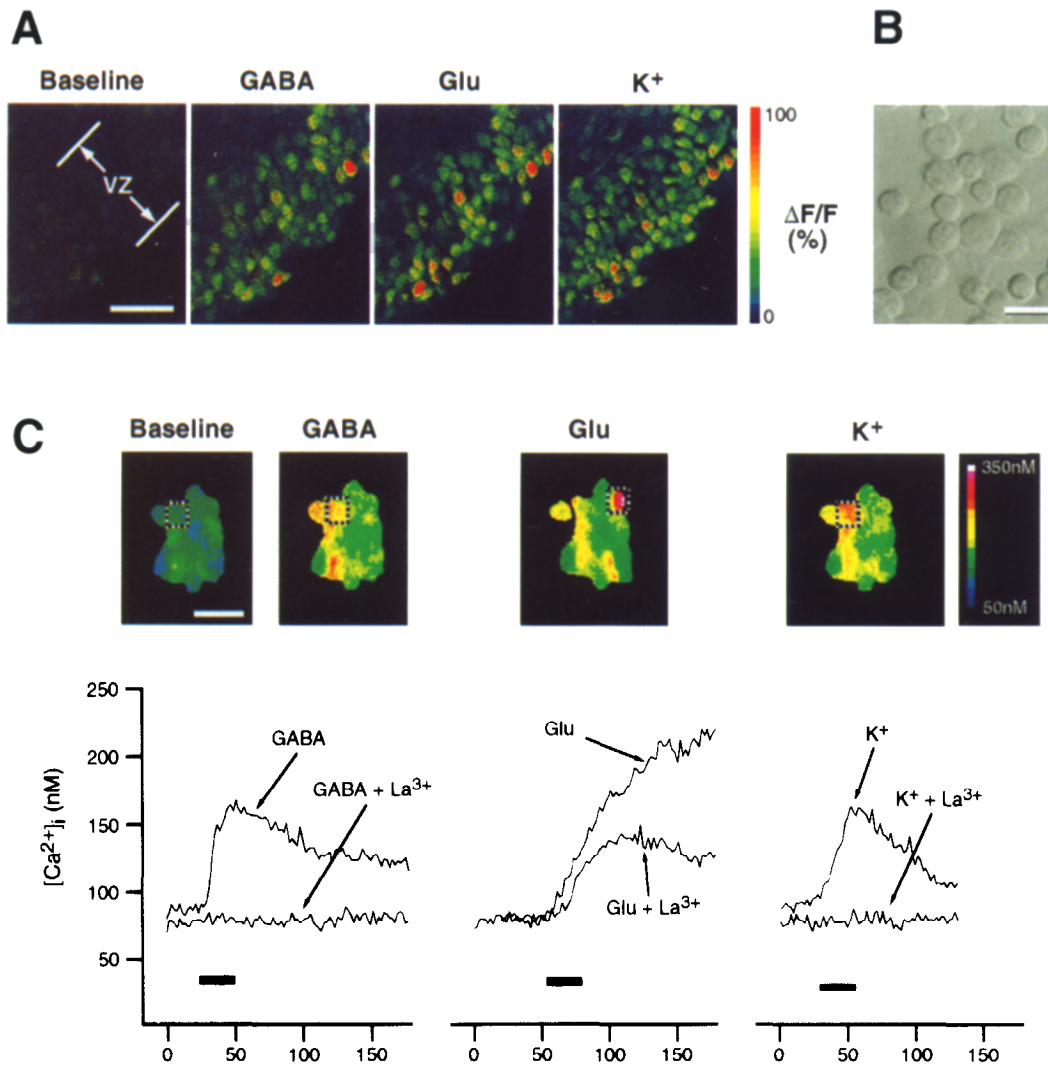

Figure 6. GABA, Glutamate, and Depolarization with KCl Increase $[Ca^{2+}]_i$ in VZ Cells

(A) Embryonic cortical cells in a coronally oriented brain slice (E16) loaded with the Ca^{2+} indicator Fluo 3 and visualized by laser confocal microscopy. The same region of embryonic cortex is illustrated immediately before drug application and 15–20 s following successive bath applications of GABA (30 μM), glutamate (Glu; 50 μM), and KCl (60 mM). The intensity of cellular fluorescence returned to control levels during a 5 min wash period between each drug application. Images show increases in $[Ca^{2+}]_i$ in VZ cells expressed as ΔF/F and are pseudocolored as indicated on the right. Bar, 40 μm.

(B) Nomarski image of a field of acutely isolated VZ cells (E16) obtained by a tissue printing technique. Cells were subsequently loaded with the ratiometric Ca^{2+} indicator Fura 2 and used in experiments in (C). Bar, 15 μm.

(C) Pseudocolored images show a field of VZ cells under control conditions (left) and at peak $[Ca^{2+}]_i$ following applications of (from left to right) GABA (30 μM), glutamate (50 μM), and KCl (60 μM) (bar, 15 μm). The responses plotted below are from the individual cells outlined in the overlying panels. When GABA and KCl are applied in the presence of La^{3+} (10 μM), the increase in $[Ca^{2+}]_i$ is abolished. The response to glutamate is partially blocked by La^{3+} (10 μM).

The physiological data reported here were derived from recordings of cells within the VZ as confirmed by dye filling. Since responses to GABA and glutamate were observed in all recordings made between the ages of E16 and E19, encompassing the peak period of cortical neurogenesis (Berry and Rogers, 1965; Hicks and D'Amato, 1968; Bisconte and Marty, 1975; Raedler and Sievers, 1976; Rickmann et al., 1977; Raedler and Raedler, 1978; Miller, 1985; Altman and Bayer, 1990), it seems likely that responsive cells included neuronal precursors. However, VZ cells are extensively coupled by gap junction channels (Lo-Turco and Kriegstein, 1991), and it is possible that SVZ cells may also be coupled to VZ cells, and thus contribute to the observed responses to GABA and glutamate. Furthermore, our DNA synthesis assay does not allow differentiation between mitotic activity in VZ or SVZ cells. At E16 in the rat, the VZ is active and generating primarily neuronal precursors, whereas the SVZ, a source of glial cells and precursors as well as some neurons, is still relatively small (Altman and Bayer, 1990). At E19 the situation is reversed: the VZ is relatively small, and the SVZ has increased in size, corresponding with an increase in glio-

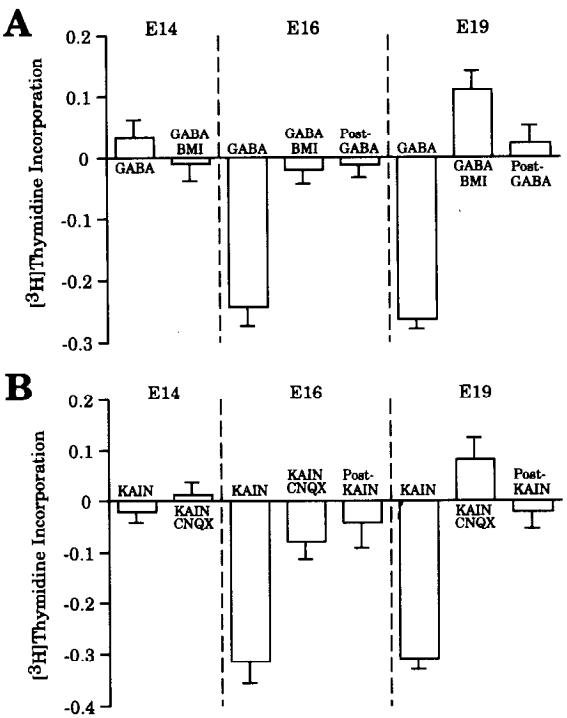

Figure 7. GABA and Kainate Decrease DNA Synthesis in Explants of Embryonic Cortex

(A) [^{3}H]Thymidine incorporation is decreased by GABA (30 μM) only after GABA receptors become functionally expressed on cortical progenitor cells (i.e., after E14). At E16 and E19, the competitive $GABA_A$ receptor antagonist BMI (50 μM) blocks the GABA-induced decrease in [^{3}H]thymidine incorporation, and [^{3}H]thymidine incorporation recovers to control levels after the removal of GABA (post-GABA). A two-way ANOVA indicated a significant effect of agonist and antagonist combination ($F(1,30) = 30.5$, $p < .05$).

(B) Kainate (150 μM) decreases [^{3}H]thymidine incorporation only after functional kainate receptors are expressed on cells in the VZ (i.e., after E14). CNQX (10 μM), a competitive AMPA/kainate receptor antagonist, blocks the kainate-induced decrease in [^{3}H]thymidine incorporation at E16 and E19, and the effects of kainate are reversible (post-KAIN) upon the removal of kainate. A two-way ANOVA indicated a significant effect of kainate versus kainate and CNQX ($F(1,30) = 27.2$, $p < .001$) and a significant interaction between age of explant and drug treatment ($F(2,30) = 4.7$, $p < .01$).

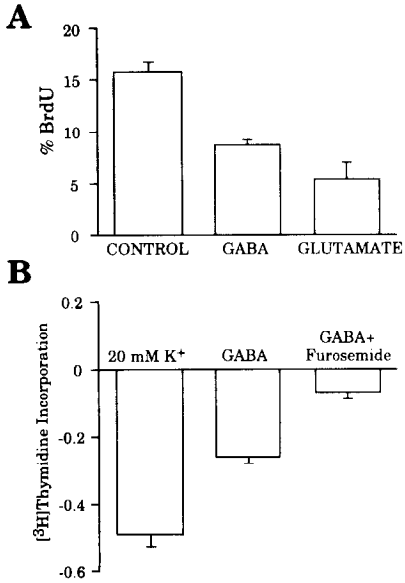

Figure 8. GABA and Glutamate Decrease the Number of BrdU-Labeled Cells, and Depolarization Alone Is Sufficient to Down-Regulate DNA Synthesis in Cortical Precursors

(A) GABA and glutamate decrease the mitotic index of cortical precursor cells as measured by the percentage of cells that incorporate BrdU. Data are from E18 explants.

(B) Depolarization is necessary and sufficient to regulate DNA synthesis in embryonic cortex. KCl (20 mM) decreases [^{3}H]thymidine incorporation as does GABA (30 μM). In the presence of furosemide (5 mM), GABA is hyperpolarizing and does not decrease [^{3}H]thymidine incorporation. Data are from E17 explants.

genesis (Berry and Rogers, 1965; Bruckner et al., 1976). As shown in Figures 8A and 8B, GABA and kainate influence DNA synthesis to a similar degree at E16 and E19. GABA and kainate may thus have similar effects on both VZ and SVZ cells and may influence both neurogenesis and gliogenesis. Our results, therefore, apply to proliferating cortical cells generally and probably include glial as well as neuronal progenitor cells.

GABA Depolarizes Proliferating VZ Cells

In proliferating cells within the VZ, GABA has a depolarizing effect, presumably owing to a high $[Cl^-]_i$. Several studies using neonatal cortical and hippocampal neurons have described depolarizing effects of GABA (Mueller et al., 1983, 1984; Janigro and Schwartzkroin, 1988; Ben-Ari et al., 1989; Swann et al., 1989), which are thought to result from a higher $[Cl^-]_i$ in immature neurons than in adult neurons. Adult hippocampal neurons possess two Cl^- transport processes, one accumulating and one extruding Cl^- (Misgeld et al., 1986). The data presented here are consistent with a proposed sequential development of Cl^- transport processes in neocortical neurons (Luhmann and Prince, 1991), with a Cl^--accumulating process predominating at early embryonic stages. This would produce a developmental decline in $[Cl^-]_i$ and account for the observed developmental shift in GABA response from depolarizing to hyperpolarizing. Our data, however, do not rule out the possibility that $GABA_A$ channels in VZ cells could also be permeable to other ions. The shift in the GABA response underscores the changing role of GABA during different stages of neuronal development.

GABA and Glutamate Receptors May Be Nonuniformly Distributed in the Proliferative Zones

The embryonic cortical cells reported here are members of coupled cell clusters within the VZ (LoTurco and Kriegstein, 1991). One consequence of the electrical coupling is that it allows the detection of current produced by the opening of even a small number of ion channels distributed over a relatively large membrane area. While this increases the sensitivity of detecting currents produced by the activation of GABA and glutamate receptors, we do not know whether the receptors are localized on the recorded cell. Similar results would be obtained whether receptors were distributed uniformly on all VZ cells or non-

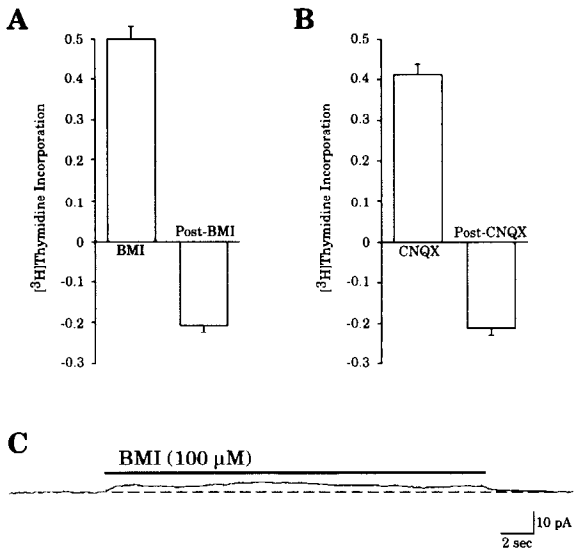

Figure 9. Endogenous Amino Acids Down-Regulate DNA Synthesis in Embryonic Cortex

(A) When E19 explants are incubated with the competitive $GABA_A$ receptor antagonist BMI (50 μM), without exogenously applied amino acids, [^{3}H]thymidine incorporation is significantly enhanced ($F(1,20) = 21.9$, $p < .001$). After the removal of BMI (post-BMI), incorporation is significantly depressed.

(B) CNQX (10 μM) increases [^{3}H]thymidine incorporation relative to controls in E19 explants; as with BMI, when CNQX is removed (post-CNQX), [^{3}H]thymidine incorporation is decreased relative to time-matched controls ($F(1,20) = 51.3$, $p < .001$).

(C) A small outward current shift is observed in VZ cells recorded in situ in response to perfusion of BMI (100 μM). This suggests tonic GABA receptor activation of these cells in situ during embryonic development. Recording shown is from an E17 VZ cell.

uniformly, perhaps localized to a subset of coupled cell types. For example, cells in several phases of the cell cycle including S phase are coupled, and it is possible that GABA and/or glutamate receptors are expressed only by cells in a particular phase of the cell cycle.

Possible Feedback Regulation of DNA Synthesis

Cells in the VZ destined for different layers undergo their last divisions in an overlapping sequence from E13 to E20 (Miller, 1988). One hypothesis for the regulation of proliferation in developing cortex is that feedback from differentiating cells terminates division in the VZ (McConnell, 1991). Since transmitter systems differentiate during early cortical development (Lauder et al., 1986; Parnavelas and Cavanagh, 1988; Van Eden et al., 1989; Blanton and Kriegstein, 1991; Schwartz and Meinecke, 1992), and since developing neurons send growth cones that can release transmitters (Taylor et al., 1990) to the top of the VZ (Kim et al., 1991), neurotransmitters are a candidate for such a feedback signal. GABAergic neurons appear in the VZ and in layer 1, the intermediate zone, and the subplate during early stages of cortical development (Marin-Padilla, 1971, 1978; Rickmann et al., 1977; Kostovic and Rakic, 1980; Raedler et al., 1980; Valverde et al., 1989; Van Eden, 1989; Cobas et al., 1991; Arimatsu et al., 1992), and they are a likely source of releasable GABA. Potential sources of releasable glutamate include cells within the VZ, growth cones from cortical plate cells, and thalamic afferents (Kim et al., 1991; Ghosh and Shatz, 1992). Transmitters released from these postmitotic cells could activate receptors on dividing cells in the VZ and regulate the timing and termination of neuronal proliferation. The effects of GABA and glutamate receptor antagonists to increase DNA synthesis in E19 explants are consistent with action on receptors on VZ cells, but DNA synthesis in the proliferative zone could be regulated indirectly through receptor blockade at another site (e.g., in the cortical plate) that in turn regulates proliferation in the VZ.

Depolarization-Induced Inhibition of DNA Synthesis

The pathway by which amino acid–induced depolarization inhibits DNA synthesis is unknown, but it is likely to involve an increase in $[Ca^{2+}]_i$ through activation of VGCCs. Our data indicate that GABA increases Cl^- conductance in VZ cells, resulting in membrane depolarization due to a high $[Cl^-]_i$. Membrane depolarization, in turn, triggers an increase in $[Ca^{2+}]_i$ through activation of VGCCs (see Figure 6). In contrast, glutamate activates AMPA/kainate receptors that depolarize VZ cells through cation flux. As in the GABA response, membrane depolarization activates VGCCs, which contribute to the increase in $[Ca^{2+}]_i$ (see Figure 6B). However, the $[Ca^{2+}]_i$ increase produced by glutamate differs from that produced by GABA because VGCCs account for only part of the glutamate-induced $[Ca^{2+}]_i$ increase. Ca^{2+} might also be able to enter VZ cells directly through glutamate-activated AMPA channels, since AMPA receptors in embryonic cortex may have a subunit composition permeable to Ca^{2+} (Hume et al., 1991; Burnashev et al., 1992). High affinity kainate receptors may also be permeable to Ca^{2+} through RNA editing of the GluR6 channel (Herb et al., 1992), though the subunit composition of kainate channels in the embryonic cortex is unknown.

The variability and relatively small sizes of the $[Ca^{2+}]_i$ transients induced by GABA and glutamate in VZ cells are consistent with a heterogeneous population of embryonic cells that express relatively few ion channels and receptors. The amplitudes of the responses to GABA and glutamate are similar to those resulting from KCl application, suggesting that the number of VGCCs may be a major determinant of the size of the $[Ca^{2+}]_i$ transient. The peak amplitude of the $[Ca^{2+}]_i$ transient is likely to depend on several factors, including the number of receptors, the Ca^{2+} permeability of the receptors, the efficiency of cytoplasmic Ca^{2+} buffering and extrusion, and the number of VGCCs. The variability in responses may reflect differences in one or more of these properties in cells at different stages of the cell cycle. Although small, the magnitude of the GABA response seen here is consistent with GABA responses reported in other embryonic cell types (Reichling et al., 1994; Obrietan and van den Pol, 1995). Small, transmitter-induced $[Ca^{2+}]_i$ transients may be a general feature of embryonic cells, suggesting that sensitive Ca^{2+}-dependent processes regulate their development.

The influx of Ca^{2+} may in turn influence progression of cortical progenitor cells through the cell cycle. At least two phases of the cell cycle, the transition of cells in G1 phase into S phase and progression through M phase, are highly Ca^{2+} dependent (Hazelton et al., 1979; Izant, 1983). Ca^{2+} and depolarization can have both positive and negative effects on cell proliferation, depending on cell type or stage of the cell cycle. For example, while depolarization by elevated K^+ or veratridine can increase DNA synthesis in cultured sympathetic neuroblasts (DiCicco-Bloom and Black, 1989), the activation of VGCCs in GH_4 pituitary cells can block G1 phase cells from entry into S phase and can also promote progression of G2 cells through mitosis (Ramsdell, 1991). Our data are consistent with GABA- and glutamate-induced regulation of the transition from G1 to S phase of the cell cycle, a transition point that is highly regulated in eukaryotic organisms (Murray and Kirschner, 1991).

Experimental Procedures

Tissue Preparation

Gravid rats (Sprague–Dawley) were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) or ketamine (50 mg/kg). Embryos were removed and immediately placed in iced ringers. Cerebral hemispheres were removed, and for experiments requiring brain slices, hemispheres were embedded in warm (38°C) 3%–4% agar (Difco, Detroit, MI) or warm (28°C–30°C) 1% low melting point agarose (Fisher Scientific, Fair Lawn, NJ) in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose), hardened on ice, and sliced with a vibratome (200–400 μm). For some experiments, telencephalic hemispheres were not sliced but prepared as slabs of neocortex by trimming off the hippocampus and striatal anlage.

Electrophysiological Recordings

Patch-clamp recordings were obtained from cells in both slices and slabs of neocortex continuously superfused with ACSF at room temperature. Methods for in situ patch-clamp recording have been described previously (Blanton et al., 1989). In brief, electrodes (5–10 MΩ) were lowered into the ventricular surface of cortical explants and slowly advanced until a resistance increase was detected (10–50 MΩ). A suction pulse was immediately applied to form a tight seal (2–40 GΩ), and additional suction was applied to rupture the underlying plasma membrane. For perforated-patch recordings (Abe et al., 1994; Kyrozis and Reichling, 1995), gramicidin (Sigma, St. Louis) was dissolved in dimethylsulfoxide (Sigma; 1–2 mg/ml) and then diluted in the pipette filling solution to a final concentration of 1–5 μg/ml. The recording techniques were as described above, except that suction was not applied after gigaseal formation. The progress of perforation was evaluated by monitoring membrane resistance and membrane potential. Drugs were applied only after the membrane resistance had reached a minimum value and membrane potential was ≤ −50 mV; this usually took 5–20 min. Patch electrodes were filled with 120 mM Cs–gluconate, 10 mM CsCl, 10 mM HEPES (pH 7.3), 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM EGTA or 100 mM CsCl, 30 mM Cs–gluconate, 10 mM HEPES (pH 7.3), 2 mM $CaCl_2$, and 10 mM EGTA (see figure legends). Some recordings were digitized and analyzed with pCLAMP (Axon Instruments, Foster City, CA). Unless otherwise noted, voltage-clamped cells were held at −60 mV.

[^{3}H]Thymidine and BrdU Incorporation Assays

For [^{3}H]thymidine incorporation experiments, explanted slabs of embryonic cerebral cortex were incubated six per 35 mm petri dish in ACSF with 5 μCi/ml [^{3}H]thymidine (Amersham, Solon, OH) at 32°C. The slabs were incubated up to 10 hr in different drug conditions and then washed in large volumes of cold, divalent cation–free ACSF for 30 min. Each slab was then solubilized in Soluene-350 (Packard, Downers Grove, IL) overnight, and trapped radioactivity was measured with scintillation spectrophotometry. [^{3}H]Thymidine incorporation was expressed as the ratio: (experimental − control)/control. For BrdU incorporation, explanted cortices were incubated in 1 μM BrdU (Sigma) for 12 hr, dissociated with trypsin, plated on slides, and processed for BrdU immunohistochemistry (Miller and Nowakowski, 1988). The fraction of cells that incorporated BrdU was determined by counting 400 cells under each condition.

Ca^{2+} Imaging in Brain Slices Using Fluo 3

Cerebral hemispheres were removed from E16 rat embryos, and 300 μm thick slices were cut on a vibratome. Cells were loaded in the dark with the Ca^{2+} indicator dye Fluo 3 by immersion for 30 min in ACSF containing the acetomethylester form of Fluo 3 (Fluo 3–AM; 10 μM) followed by ACSF wash. Slices were attached to a coverslip and placed in a perfusion chamber on the stage of a Zeiss Axiovert microscope (40×, NA 0.75 objective) with a Bio-Rad MRC-600 argon laser scanning confocal attachment. Excitation was at 488 nm light, and emissions were collected using a 515 nm long-pass emission filter. Neutral density filters were used to filter the argon laser light to 1% to minimize photobleaching. Images were acquired at 1.0 s/frame, and three frames were averaged for each image. Fluorescence micrographs were digitized and data expressed as a change in fluorescence over baseline fluorescence (ΔF/F). The VZ was visualized and perfused with drug-free medium, while three images were averaged to obtain the baseline value F. Each subsequent image during drug application and washout was processed and expressed as a change in fluorescence over baseline fluorescence (F) by ΔF/F. Images were acquired on an IBM compatible computer running Comos acquisition software (Bio-Rad, Hercules, CA). Images were analyzed and pseudocolored using NIH Image software on a Macintosh computer.

Ca^{2+} Imaging in Isolated VZ Cells Using Fura 2

The neocortex was surgically removed from E16 embryos and dissected into relatively flat fragments (1–2 mm^2). VZ cells were transferred to coverslips by a tissue printing technique. Each fragment was rinsed and placed ventricular side down against the surface of a poly-D-lysine-coated coverslip held in a 35 mm plastic petri dish (Falcon). Each cortical fragment was briefly and gently pressed against the coverslip by aspirating away enough fluid to flatten the cortex for 5 s. The cortical fragment was then removed, leaving cells adherent to the surface of the coverslip. The coverslip-containing petri dishes were then washed and filled with buffered saline (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, 19 mM sucrose, 5.5 mM glucose; pH 7.3) containing 10 mM Fura 2–AM and placed at room temperature in the dark for 30 min to permit intracellular loading with dye. The coverslips were then rinsed and transferred to a perfusion chamber on the stage of an inverted epifluorescence-equipped microscope (Zeiss Axiovert 135 TV). Drugs were applied by bath perfusion.

Fura 2 was excited by 75W Xenon bulb emission passed through 340 and 380 nm filters mounted in a Sutter filter wheel (Novato, CA). A fiberoptic cable was used to mechanically isolate the filter wheel from the microscope. Emission fluorescence was intensified by a Hamamatsu image intensifier and collected by a Hamamatsu CCD camera, both mounted beneath the microscope. Images were digitized and processed by a Videoprobe system (ETM Systems, Irvine, CA), which also controlled the filter wheel. Background values were collected at each wavelength from a region between cells or from a cell-free field. $[Ca^{2+}]_i$ was computed on-line using the equation:

$$[Ca^{2+}]_i = K_D \times S_f \times (R - R_{min})/(R_{max} - R),$$

where K_D is the apparent dissociation constant of Fura 2 for Ca^{2+}, S_f is the scale factor of the optical system, R is the measured ratio of fluorescence at 340 and 380 nm, R_{min} is the fluorescence ratio in the presence of zero Ca^{2+}, and R_{max} is the fluorescence ratio in the presence of a saturating (1 mM) concentration of Ca^{2+}. A series of EGTA-buffered solutions containing standardized concentrations of Ca^{2+} and 100 μM Fura 2 pentapotassium salt was used to calibrate this system. The solutions were placed in 20 μm precision path length rectangular capillary tubes, and background-subtracted ratiometric measurements were obtained. This in vitro calibration protocol gave an appar-

ent K_D of 242 nM, which agrees with previously described values (Grynkiewicz et al., 1985).

Intracellular Labeling Experiments

In some experiments, a saturated solution of the fluorescent dye Lucifer yellow dipotassium salt (Molecular Probes, Eugene, OR) was added to the electrode filling solution used to fill the tips of the electrodes. For the combination of autoradiography and intracellular labeling, cells were filled for 20 min with 1%–2% biocytin and incubated in 5 μCi/ml [^{3}H]thymidine at 32°C for 1 hr. Tissue was then fixed in 4% paraformaldehyde, reacted to visualize biocytin (Horikawa and Armstrong, 1988), embedded in paraffin, sectioned, and mounted on slides. The slides were dehydrated in graded alcohol, air dried, and dipped into NTB2 nuclear track emulsion (Kodak, Rochester, NY) diluted 1:1 with water, exposed for 4–7 days in the dark at 4°C, developed, dehydrated, and coverslipped.

Pharmacological Agents

GABA, glutamate, kainate, muscimol, furosemide, BMI, glycine, carbachol (Sigma), CNQX, AMPA, APV, NMDA (Tocris, Bristol, England), baclofen, and picrotoxin (RBI, Natick, MA) were applied by bath perfusion or by focal application (DAD-12 Superfusion System, ALA Scientific Instruments, Westbury, NY). Qualitatively similar results were obtained for both application procedures. When using focal application, an ACSF wash was applied immediately after drug application. For DNA synthesis assays, explants were transferred in a minimal volume to 2 ml of medium containing the indicated drugs. All drugs were kept as concentrated stock solutions at −20°C and diluted to the desired concentration on the day of the experiment.

Acknowledgments

We thank Beth Armitage, Steven Siegelbaum, and Stephen Rayport for helpful comments in the preparation of this manuscript, Alex Flint for help with computer graphics, and John Avilla for technical assistance. This work was supported by National Insitute of Neurological Diseases and Stroke grant NS 21223 to A. R. K., National Institutes of Health epilepsy training grant NS 07280 to J. J. L., and the Foundation for Anesthesia Education and Research with a grant from Abbott Laboratories for M. J. S. H.

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Received April 19, 1995; revised August 15, 1995.

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