GABA\textsubscript{B} Receptors Mediate Motility Signals for Migrating Embryonic Cortical Cells

During development, postmitotic neurons migrate from germinial regions into the cortical plate (cp), where lamination occurs. In rats, GABA is transiently expressed in the cp, near target destinations for migrating neurons. In vitro GABA stimulates neuronal motility, suggesting cp cells release GABA, which acts as a chemoattractant during corticogenesis. Pharmacological studies indicate GABA stimulates migration via GABA\textsubscript{B}-receptor (GABA\textsubscript{B}-R) activation. Using immunohistochemistry, RT-PCR and Western blotting, we examined embryonic cortical cell expression of GABA\textsubscript{B}-Rs in vivo. At E17, GABA\textsubscript{B}R1\textsuperscript{c} cells were identified in the ventricular zone (vz) and cp. RT-PCR and Western blotting demonstrated the presence of GABA\textsubscript{B}R1\textsuperscript{a} and GABA\textsubscript{B}R1\textsuperscript{b} mRNA and proteins. Using immunocytochemistry, GABA\textsubscript{B}-R expression was examined in vz and cp cell dissociates before and after migration to GABA in an in vitro chemotaxis assay. GABA-induced migration resulted in an increase of GABA\textsubscript{B}-R\textsuperscript{c} cells in the migrated population. While <20% of each starting dissociate was GABA\textsubscript{B}-R\textsuperscript{c}, >70% of migrated cells were immunopositive. We used a microchemotaxis assay to analyze cp cell release of diffusible chemotropic factor(s). In vitro, cp dissociates induced vz cell migration in a cell density-dependent manner that was blocked by micromolar saclofen (a GABA\textsubscript{B}-R antagonist). HPLC demonstrated cp cells release micromolar levels of GABA and taunrine in several hours. Micromolar levels of both molecules stimulated cell migration that was blocked by micromolar saclofen. Thus, migratory cortical cells express GABA\textsubscript{B}-Rs, cp cells release GABA and taunrine, and both molecules stimulate cortical cell movement. Together these findings suggest GABA and/or taunrine act as chemoattractants for neurons during rat cortical histogenesis via mechanisms involving GABA\textsubscript{B}-Rs.

Introduction

During central nervous system (CNS) development, neuroblasts proliferate in germinial zones, undergo terminal mitosis, then migrate to their final positions. Recent studies indicate that the amino acids GABA and glutamate influence neuronal migration in many developing brain regions including the cerebellum (Komuro and Rakic, 1993), the olfactory placode (Tobet et al., 1996; Fueshko et al., 1998), the spinal cord (Behar et al., 1994), and the cortex (Behar et al., 1996, 1998, 1999; Marret et al., 1997). These studies demonstrate that a single amino acid can either promote or arrest cell movement by activating different classes of receptors.

In the developing rat cortex, GABA is transiently expressed near target destinations for migrating neurons and embryonic cortical neurons exhibit migratory responses to GABA in vitro (Lauder et al., 1986; Behar et al., 1996, 1998, 1999). Suggesting GABA is a chemoattractant during corticogenesis, GABA modulates cortical neuronal movement via multiple classes of receptors. In the mature CNS, three classes of GABA receptors have been described. Ionotropic GABA\textsubscript{A} and GABA\textsubscript{B} receptors (GABA\textsubscript{A}Rs and GABA\textsubscript{B}Rs) form Cl\textsuperscript{−} channels (Sivilotti and Nistri, 1991; Feigenspan et al., 1993); metabotropic GABA\textsubscript{B} receptors (GABA\textsubscript{B}Rs) couple to Ca\textsuperscript{2+} or K\textsuperscript{+} channels or adenylate cyclase via G\textsubscript{i} or G\textsubscript{o} GTP binding proteins (Kerr and Ong, 1992). Pharmacological studies indicate GABA\textsubscript{B}R agonist receptor activation stimulates migration of neurons in immature cortical regions, while GABA\textsubscript{A}R activation arrests migration as neurons approach their target destinations in the cortical plate (cp) (Behar et al., 1998).

In vitro migration studies on acutely dissociated cells demonstrated that GABA's pro-migratory signals were mimicked by the GABA\textsubscript{B}R agonist, baclofen (Kerr and Ong, 1992; Behar et al., 1998). Femtomolar GABA or baclofen stimulated gradient-dependent or directed migration (chemotaxis) of neurons from immature cortical regions (vz), while micromolar levels induced gradient-independent or random motility (chemokinesis) of neurons isolated from the cp. Migration was blocked by the GABA\textsubscript{B}R antagonist, saclofen (Kerr and Ong, 1992), and by pertussis toxin, an inhibitor of G\textsubscript{i}, G\textsubscript{o} GTP binding proteins (UI, 1984), implying that GABA\textsubscript{B}R activation mediates pro-migratory signals in both populations (Behar et al., 1998).

The effects of GABA\textsubscript{B}R activation on embryonic cell migration were confirmed in experiments using cultured cortical slices. Application of saclofen to cultured slices inhibited cells in the intermediate zone (iz) from migrating into the cp (Behar et al., 2000). Since exogenously applied attractants were not added to the cultures, the results implied that cortical cells in situ released diffusible factors that directed cells in the iz to migrate into the cp by activating GABA\textsubscript{B}R-like receptors.

GABA\textsubscript{B}Rs are heteromeric receptors consisting of GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 subunits (Jones et al., 1998; Kaupmann et al., 1998a; White et al., 1998; Kuner et al., 1999). Two splice variants of the GABA\textsubscript{B}R1 receptor (R1a and R1b) have been identified (Kaupmann et al., 1997), which apparently exhibit different anatomical distributions and functions (Kaupmann et al., 1998a; Benke et al., 1999; Bischoff et al., 1999). Presynaptic GABA\textsubscript{B}Rs inhibit neurotransmitter release by down-regulating voltage-activated Ca\textsuperscript{2+} channels, whereas postsynaptic GABA\textsubscript{B}Rs decrease neuronal excitability by activating an inwardly rectifying K\textsuperscript{+} (Kir3) conductance (Kaupmann et al., 1998b). To form receptors with native physiological properties, expression of both GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 subunits are required (Jones et al., 1998; Kaupmann et al., 1998b; White et al., 1998; Kuner et al., 1999). The pharmacology of migratory responses to GABA implied embryonic rat cortical cells express GABA\textsubscript{B}R-Rs. Here, we used immunocytochemistry, RT-PCR and Western blotting to examine the expression of GABA\textsubscript{B}Rs in the rat embryonic cortex in vitro. Using an in vitro chemotaxis assay (Behar et al., 1994), we analyzed whether cp cells release diffusible factors that induce vz cells to migrate. HPLC was used to measure the release of GABA and other amino acids from vz and cp cells. Our findings indicate that cp cells stimulate vz cells to migrate, and cp cells release GABA and taunrine, both of which act as...
Materials and Methods

Dissection and Dissociation of Cortical Cells
Timed pregnant Sprague-Dawley mothers at embryonic day 18 (E18) were killed with CO₂. The embryos were removed by cesarean section. Brains from the embryos were dissected and placed into Leibovitz's L-15 Medium (Life Technologies, Grand Island, NY, USA) supplemented with 0.05 mg/ml gentamycin. Whole cortices were cleaned of meninges and removed. For the ventricular zone (vz) and cp microdissections, 350 µm coronal slices of brain were prepared using a McIlwain tissue chopper (Brinkman, Westbury, NY, USA). The slices were placed in a tissue containing cold L-15 medium and were microdissected through the i.z to obtain vz and cp tissue segments. The vz segment included the vz, subventricular zone (svz) and lower half of the i.z. The cp segment included the cp, subplate (sp) and upper half of the i.z. Preparation and characterization of the vz and cp dissociates has been detailed elsewhere (Behar et al., 1998).

Briefly, whole cortices or vz or cp tissue segments were dissociated enzymatically into single-cell suspensions with papain (20 U/ml, Worthington Biochemical Corp., Freehold, NJ, USA). 0.005% DNase (Boehringer Mannheim, Indianapolis, IN, USA), 0.5 mM EDTA (Life Technologies), and 1 mM L-cysteine at 37°C for 10 min. The cell suspension was trituated through a 10 ml pipette, spun at 300 g for 5 min, and resuspended in Earle’s Balanced Salt Solution (EBSS, Life Technologies) containing 1 mg/ml bovine serum albumin (BSA) and 1 mg/ml ovomucoid trypsin inhibitor (Sigma Chemical Corp., St Louis, MO, USA). The cell suspension was layered over 5 ml of EBSS containing 1 mg/ml each of BSA and trypsin inhibitor and was centrifuged at room temperature at 80 g for 7 min. Cells were resuspended in EBSS containing 16 mM glucose at a concentration of 1 x 10⁶/ml for immunocytochemistry and the chemotaxis studies.

HPLC Analysis of Amino Acid Release from Dissociated Cells
vz and cp tissue segments from E17 cortices were dissociated into single-cell suspensions as described above. One million vz or cp cells were resuspended in 1 ml of Medium A (145 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 10 mM HEPES buffer, 10 mM glucose; pH 7.2) and incubated at 37°C for 30 min. The cells were pelleted at 80 g for 10 min, and the supernatants were collected and stored at -20°C until analyzed by HPLC. To verify that the cells did not lyse during the 30 min incubation period, the viability of the cells was assessed using trypan blue exclusion on an aliquot of each dissociate before and after the incubation at 37°C. Prior to the incubation, the viability of the vz cells was 98.8% (±2.1%); cp cell viability in the starting dissociate was 99.4% (±0.9%). After the incubation, the viability of the vz cells was 99.9% (±1.5%); cp viability was 98.9% (±1.0%), confirming that there was no significant death of the cells due to lysis during the 30 min incubation period.

Amino acids were determined using capillary liquid chromatography with electrochemical detection as described previously (Boyd et al., 2000). Briefly, 2 µl aliquots of samples were derivatized by adding 0.4 µl of 40 mM o-phenthalddehyde (OPA) and 50 mM tert-butyl thiol in 50% 40 Mborate at pH 10.5/250 mM NaOH and allowing to react for 2 min. Samples were diluted as necessary with HPLC grade water.) The reaction was quenched by adding 0.4 µl of 1 M iodacetamide in methanol and allowed to react for 4 min. Immediately after derivatization, 100 µl of the sample was injected onto a capillary liquid chromatography column (48 µm i.d. x 36 cm long fused silica capillary packed in-house with 5 µm, 100 nm pore Alltiima C8 particles). All derivatization and injection procedures were performed using a FAMOS autosampler (LC Packings, Amsterdam, The Netherlands). Mobile phase for the separation consisted of a 55% A: 45% B for 1 min with a linear increase in B to 100% over 8.5 min where A was 50 mM phosphate buffer at pH 6.5 and B was 55% 50 mM phosphate buffer at pH 6.5 and 65% CH₃CN. Mobile phase flow rates were 350 n/min. Separated analytes were detected amperometrically using a carbon fiber microelectrode (9 µm diameter x 1.5 mm length) poised at +750 mV versus Ag/AgCl. Significance of the level of each amino acid spontaneously released from vz cells was compared with the level of the same amino acid released from cp cells using Student’s t test.

Immunohistochemistry of Sections
E17 rat brains were dissected and fixed in 4% paraformaldehyde (PF) for 4 h, cryoprotected in 30% sucrose at 4°C for 3 days and frozen in liquid nitrogen-cooled isopentane. Using a cryostat, 20 µm thick coronal sections were cut, dried at room temperature for 1 h and incubated overnight at room temperature in guinea pig anti-GABAᵩ1 receptor antibody (1:5000; Chemicon International, Temecula, CA, USA). Slides were washed three times in phosphate-buffered saline (PBS), pH 7.2, and incubated for 1 h at room temperature in biotinylated donkey anti-guinea-pig IgG (Jackson ImmunoResearch, West Grove, PA, USA). All antibodies were diluted in PBS containing 10% normal rat serum and 10% normal donkey serum to prevent nonspecific binding. The slides were washed three times in PBS, reacted with streptavidin-conjugated horseradish peroxidase (Jackson ImmunoResearch) and developed in AEC substrate (25 mg AEC in 100 ml of acetate buffer) (Sigma) with 0.01% H₂O₂ for 10–15 min at room temperature. Control sections incubated in diluent only, or in normal guinea-pig serum (1:5000) in lieu of primary antibody, exhibited no immunoreaction product.

Immunocytochemistry of Cells
Fifty thousand dissociated vz or cp cells from E18 cortices (50 µl of a 10⁷/ml cell suspension) were seeded onto 35 mm culture dishes pre-coated with poly-o-lysine (>450 000 mol. wt, 20 µg/ml) (Collaborative Research, Bedford, MA, USA) and incubated at 37°C for 1 h. Cells were fixed in 4% PF for 50 min, then incubated overnight at 4°C in guinea-pig anti-GABAᵩ1 receptor antibody (1:1000; Chemicon). Cells were washed three times in PBS, then incubated for 1 h at room temperature in peroxidase-conjugated donkey anti-guinea-pig IgG (1:200, Jackson ImmunoResearch). Antibody staining was visualized with diaminobenzidine (DAB) substrate. When diluent only or normal guinea-pig serum (1:1000) was used in place of anti-GABAᵩ1 antibody, cells did not immunostain.

RT-PCR
A hot-start RT-PCR (reverse transcription-polymerase chain reaction) protocol was used to measure gene expression. The sequences were as follows:

GABAᵩ1af: TGCTGACACCTTGCCAACCC
GABAᵩ1ar: GTGACATAGGGCCATAGCCACAG
GABAᵩ1bf: ACGCATCAGCGCCACAG
GABAᵩ1br: AACCAGTGGTCACTACATACACCC

Total RNA was isolated from E17 cortical cp and vz tissue homogenates RNAstat 60 (Tel-Test, Inc., Friendswood, TX, USA) and its recommended protocol was used for the RNA isolation. To confirm purity of the product RNA, absorption ratios at 260 nm/280 nm were determined to be >1.8 for all samples. The samples were adjusted to 200 ng/µl for reverse transcription and PCR according to absorption at 260 nm. PCR (Perkin Elmer GeneAmp RNA PCR kit, Applied Biosystems) involved preheating a mixture of Taq antibody (TaqStart, Clontech, Palo Alto, CA, USA), primers, cDNA and PCR components to 97°C for 90 s before amplification. PCR cycle was 30 s at 95°C (denaturation), 45 s at 60°C (annealing) and 60 s at 72°C (extension). Amplification was within the exponential range. Control RNA (transcribed from cDNA, transcription and PCR amplification reactions permitted detection of inefficient PCR reactions.

To control for artifacts due to DNA contamination, in some studies, RT-PCR was carried out without reverse transcriptase. In the absence of reverse transcriptase, no bands were visualized. In addition, RNA samples in some studies were verified to be free of DNA contaminants as described previously (Somogyi et al., 1995).

To verify the identity of PCR products, restriction digests were performed using restriction endonucleases targeted to known internal sequences to produce a distinctive pattern of restriction products. A 5 µl volume of PCR product from RT-PCRs was subjected to an additional 30-cycle PCR using the above parameters. The reamplified DNA was purified using the Magic PCR Preps kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. A 8 µl volume of each
fragment was mixed with 1 µl of 10× digestion buffer and 10 µl of restriction endonuclease solution, then incubated for 1 h at 37°C. For both fragments, the generated restriction fragments appeared at their predicted positions.

Migration Studies

Dissociated cells from either E17-18 whole cortices or vz and cp tissue segments were segmented to GABA or taurine (Sigma) using an in vitro chemotaxis assay (Neuroprobe, Inc., Cabin John, MD, USA) described previously (Behar et al., 1994, 1998). Attractants were diluted in EBSS plus glucose. Prior to assembly, the membrane was coated with poly-L-lysine (>450000 mol. wt; 50 µg/ml; Collaborative Research, Bedford, MA, USA) to promote adhesion. Cells (50 000) were incubated in the presence of attractants or cortical dissociates for 18 h at 37°C with 10% CO2 and 90% air. Following the incubation, the chambers were disassembled and the cells on the upper surface of the membrane that failed to migrate were scraped off. Migrated cells on the underside of the membrane were fixed, stained and counted as described below.

Quantification of Migration

For quantitative assays, migrated cells were fixed 30 min in 4% PF with 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and stained 5 min in 0.1% cresyl violet. The membranes were mounted cell-side up onto 2 inch × 3 inch glass slides, allowed to air dry, and covered with immersion oil. Cells were counted under bright field using oil immersion 16× or 40× Zeiss Planapo objectives on a Zeiss photomicroscope. Only cells that passed completely through the pores and migrated out onto the underside surface of the membrane were considered migrated (positive) cells. Spontaneous (random) migration of cells in control wells containing buffer only was also assessed in all assays. Each attractant condition was run in triplicate wells and 5–10 fields of stained cells were counted for each well. The average number of migrated cells/mm² for each attractant condition was calculated and the statistical significance was analyzed using ANOVA followed by Fisher’s PLSD test. Unless noted, illustrations are representative plots from individual experiments. Spontaneous migration in control wells containing buffer only ranged from 0 to 5 cells/mm².

Western Blotting

E17 whole cortex and adult cortical tissue were dissected, homogenized, and 30 µg of protein from each homogenate was separated by electrophoresis on 8–16% polyacrylamide gradient gels (Novex, Encenitas, CA, USA). Proteins were transferred to nitrocellulose sheets, and the blots were also stained and counted as described below.

GABA<sub>B</sub>R1 proteins were visualized using guinea-pig anti-GABA<sub>B</sub>R antibody (1:100, Chemicon). The anti-GABA<sub>B</sub>R antibody recognizes both the R1a and R1b subunit proteins. Affinity-purified, alkaline phosphatase-conjugated donkey anti-guinea-pig second antibodies (1:500, Jackson ImmunoResearch) were used to visualize protein bands. Blots were incubated in substrate solution (Bio-Rad, Hercules, CA, USA) to reveal the presence of immunoreaction products. Molecular weight standards (Novex) were run with each sample; blots were run in three separate trials on tissue homogenates prepared from three separate litter groups.

Migration in Cultured Cortical Slices

The slice culture method for studying cell migration has been detailed previously (Behar et al., 1999). Briefly, E18 brains from littermates were collected and maintained in media containing antagonist (100 µM 2-hydroxy-saclofen, RBI, Natick, MA, USA). Antagonist was added at high micromolar levels to ensure that it penetrated through the thick sections. Slices were cultured at 37°C in a mixture of 10% CO2 and 90% air. Viability of cells in the cultured slices was verified as previously described (Behar et al., 1999).

BrDU Immunolabeling

Two days after removing BrDU from the cultures, the slices were fixed for 1 h in 4% PF, then permeabilized for 1 h in 0.5% Triton X-100. After rinsing in PBS, the slices were then incubated for 10 min in cold (4°C) 1 N HCl, then incubated for 25 min at 37°C in 1 N HCl. The HCl was removed and the pH of the slices was neutralized by adding 0.1 M Tris–HCl buffer, pH 8.0. The slices were then rinsed twice in PBS and incubated overnight at 4°C in the anti-BrDU antibody (diluted 1:53 in PBS) (Boehringer Mannheim). Immunolabeling for BrDU was visualized using the Elite Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and DAB. Following immunostaining, slices were briefly (30 s) counterstained with 0.05% cresyl violet for visualization of cytoarchitectural features. Stained sections were transferred onto glass slides and coverslipped.

Density of BrDU-labeled Cells by Cortical Region

BrDU is incorporated into proliferating cells during DNA synthesis or S phase of the cell cycle. Therefore, it remains permanently integrated in the DNA and can be detected at any point during a cell’s lifespan. If a cell incorporates BrDU during its terminal mitosis, all of the BrDU remains within that cell’s DNA, and the subsequent immunostaining for the marker appears as a dense dark brown label in the nucleus. However, when a cell that has incorporated BrDU during a pulse undergoes further proliferation, the BrDU becomes distributed among the nucleic DNA of the daughter cells, thus the BrDU immunoreaction signal is ‘diluted’. In these cells, the immunostaining appears paler in comparison to the cell incorporating BrDU during terminal mitosis. In these studies, only dense, dark brown nuclei, which were interpreted as representing cells that underwent terminal mitosis during the BrDU pulse, were considered positive and analyzed by densitometry. Cresyl violet staining allowed for the identification of the ventricular zone/subventricular zone (v/z/svz), intermediate zone (iz) and cortical plate (cp) compartments. The pixel density in each cortical compartment was measured, and the compartmental contribution to the total pixel density for the slice (% of total BrDU’s pixel density by region) was calculated. A total of 30 measurements on six coronal slices from three different litters were collected and averaged for each trial. Illustrations present the averages (±SEM) from five trials.

Results

Embryonic Cortical Cells Express GABA<sub>B</sub>R Receptors In Vivo

E17 cortical sections were immunolabeled with anti-GABA<sub>B</sub>R antibody to identify cells expressing GABA<sub>B</sub>R1 proteins in the embryonic cortex. Immunopositive cells were observed in the ventricular and subventricular regions, as well as in the cortical plate (Fig. 1). High magnification revealed that immunoreactivity was evident in both the soma and neurites of the immunopositive cells. Occasional immunolabeled unipolar cells with dendrites that were evident in both the soma and neurites of the immunopositive cells. Occasional immunolabeled unipolar cells with dendrites that were evident in both the soma and neurites of the immunopositive cells. Occasional immunolabeled unipolar cells with dendrites that were evident in both the soma and neurites of the immunopositive cells. Occasional immunolabeled unipolar cells with dendrites that were evident in both the soma and neurites of the immunopositive cells. Occasional immunolabeled unipolar cells with dendrites
throughout the cortex express GABA\(_B\)-R1 proteins; however, the commercially available antibody used in these studies does not discriminate between the R1a and R1b splice variants.

To verify that embryonic cortical cells express mRNA encoding GABA\(_B\)-R1 receptors and to identify the splice variants expressed by embryonic cortical cells, E18 cortices were microdissected into two tissue segments that effectively separates the immature cells of the ventricular zone/subventricular zone (vz) from the more mature neurons of the cortical plate/subplate (cp) (Behar et al., 1998). The vz and cp tissue segments were then analyzed by RT-PCR for the expression of mRNA encoding R1a and R1b subunits. The PCR studies demonstrated the presence of transcripts of predicted lengths (763 and 347) corresponding to mRNA encoding R1a and R1b subunits in tissue homogenates from each cortical region (Fig. 2A,B), providing further evidence that embryonic cortical cells express GABA\(_B\)-Rs in vivo. Western blotting with the anti-GABA\(_B\)-R1 antibody confirmed the presence of two immunoreactive protein bands (130 and 100 kDa), corresponding to R1a and R1b proteins respectively (Malitschek et al., 1998) (not shown).

To determine if the cells that migrate to GABA express GABA\(_B\)-R proteins, vz and cp tissue segments were dissociated into single-cell suspensions and the cells in each dissociate were immunolabeled with anti-GABA\(_B\)-R antibodies before and after migration. While <10% of the starting vz dissociate was GABA\(_B\)-R+, >70% of the vz cells that migrated to micromolar GABA were immunopositive. Similarly, <20% of the starting cp population was GABA\(_B\)-R*, however, nearly 70% of the cp cells that migrated to micromolar GABA were immunoreactive (Fig. 3E). Thus, migration to GABA appeared to result in an enrichment of GABA\(_B\)-R* cells; while few cells in the starting populations were GABA\(_B\)-R*, over two-thirds of the cells that migrated to each GABA concentration expressed GABA\(_B\)-R1 proteins.

**Figure 1.** GABA\(_B\)-R1 receptor expression in vivo. E17 cortical sections were immunostained with anti-GABA\(_B\)-R1 antiserum. (A) Immunopositive cells are evident in the cortical plate and ventricular zone regions of the cortex. High magnification reveals that immunoreactivity is distributed in both the soma and neurites of the cells in the cortical plate (B) and ventricular zone (C). (D) An enlarged, expanded view of the section shown in (B) [white boxes in (B) and (D) indicate identical regions] reveals GABA\(_B\)-R1 + cells in the intermediate zone that exhibit the typical unipolar morphology of migrating cells (arrowheads). bv = blood vessel, cp = cortical plate, iz = intermediate zone, LV = lateral ventricle, vz = ventricular zone, I = layer I. Bar in (A) = 100 \(\mu\)m and in (B-D) = 50 \(\mu\)m.

**Figure 2.** Identification of the GABA\(_B\)-R1 receptor splice variants expressed in the E18 cortex. RT-PCR demonstrates the presence of transcripts encoding R1a (A) and R1b proteins (B) in tissue homogenates of adult cortex, cortical plate tissue segments (cp) and ventricular zone tissue segments (vz).

**Blockade of GABA\(_B\) Receptors Retards Cell Migration in Cultured Slices**

The studies on the dissociated cells demonstrated that direct application of GABA stimulated migration of dissociated embryonic cortical neurons. To determine if attractants generated by cells within the cortex influence cell motility, we analyzed the effect of saclofen on cellular migration in organotypic cortical slice cultures (Behar et al., 1999). In the developing cortex, the proliferating cells are located in the ventricular and subventricular zones (Jacobson, 1991). Since proliferating cells incorporate BrdU, we exposed the cultured slices to a pulse of BrdU, then used changes in the anatomical distribution of the BrdU-labeled cells in the slices over time as an indication of postmitotic cell migration.

After 2 days of culture, densitometry revealed that nearly 80% of the BrdU-labeled cells were localized in the cortical plate (cp) and subplate (sp) of the control slices (Fig. 4A,C). In controls, the ventricular and intermediate regions contained less than 20% of all BrdU-labeled cells. In contrast, antagonist-treated contralateral slices maintained for 48 h in the presence of saclofen had few labeled cells in the cp (Fig. 4B,C). Most BrdU-positive cells (>80%) in the treated slices were found in the ventricular and intermediate zones (Fig. 4C). These results demonstrate that a
48 h exposure to saclofen inhibited the migration of postmitotic vz cells into the cp.

After 6 days of culture, the distribution of BrdU+ cells had changed in slices maintained under both conditions. In controls, all of the BrdU+ cells were found in the cp. In contrast, only 70% (±2%) of BrdU+ cells were found in the cp of the saclofen-treated slices. When antagonist was present, nearly 30% of all BrdU+ cells were still evident in the iz after 6 days, however the vz was virtually devoid of labeled cells (Fig. 4D). These results indicate that blocking GABA-B-Rs with saclofen delayed, but did not completely arrest, postmitotic cell migration into the cp.

**cp Cells Release a Diffusible Factor that Stimulates vz Cells to Migrate**

Since attractants were not added to the slice cultures, the results of the slice culture studies suggested that cortical cells release a factor(s) that induces postmitotic vz cells to migrate into the cp. To assess whether cortical cells release a diffusible factor that directly stimulates migration, we placed a fixed number of dissociated cortical cells (50,000) in the upper half of the chemotaxis chamber, opposite wells containing decreasing densities of cortical dissociates. We found that dissociated cortical cells in the bottom of the chamber induced the cells in
the upper half of the chamber to migrate in a density-dependent manner that paralleled the bimodal dose–response relationship observed when cortical cells were exposed to different concentrations of exogenous GABA (Fig. 5A, B) (Behar et al., 1996). GABA induces peak levels of migration of E17 cortical cells at concentrations of 500 nM and 500 fM (Behar et al., 1996). A bimodal distribution with relative peaks was observed when 90 000 or 750 000 cells were placed in the lower wells of the chamber (Fig. 5B). At intermediate concentrations of exogenous GABA or densities of cell dissociates, insignificant levels of migration were observed (Fig. 5B).

These results suggest that cp cells release either GABA, or another diffusible factor that induces vz cells to migrate. Thus, we dissected embryonic cortices into vz and cp tissue segments, dissociated the segments into single-cell suspensions, and analyzed the migratory responses of 50 000 vz cells in the upper half of the chamber to various concentrations of dissociated cp cells that were placed in the lower wells. We found that 200 000 cp cells in the lower wells induced vz cell migration at levels similar to those observed when vz cells were migrated to 500 fM GABA (Fig. 5C). Furthermore, the vz cell migration induced by the cp cells was blocked >60% in the presence of 10 µM saclofen (Fig. 5C). These results demonstrate that cp cells release a diffusible factor that stimulates the migration of a subset of vz cells via a mechanism that involves activation of GABAβRs.

**Cortical Cells Release Micromolar Levels of Amino Acids**

Since the chemotaxis assay provided evidence that cortical cells release either GABA or other diffusible chemotropic factors, HPLC was used to measure the levels of five amino acids released spontaneously from vz and cp cells suspended at the same density (Table 1). Of the five amino acids analyzed, there were elevated concentrations of three in the cp supernatant compared with the vz supernatant. The level of taurine was 3.3-fold greater in the cp supernatant compared with the vz supernatant ($P \leq 0.05$). Similarly, the cp supernatant contained elevated levels of aspartate (2.2-fold) and GABA (3.1-fold) compared with the supernatant from the vz cells ($P \leq 0.05$). In contrast, the level of two other amino acids, β-alanine and glutamate, did not differ in the two supernatants. These results indicate that cp cells spontaneously release taurine, aspartate and GABA, and provide evidence that in vivo, there may be a concentration gradient of these amino acids in the cp relative to the vz. Viability studies on the incubated cells confirmed that

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<th>Amino acid</th>
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<tr>
<td>Asp</td>
<td>vz</td>
<td>0.51 ± 0.18</td>
<td>1.19 ± 0.47*</td>
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<td>Cp</td>
<td>0.43 ± 0.05</td>
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<td>Glu</td>
<td>vz</td>
<td>0.38 ± 0.09</td>
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<td>β-Ala</td>
<td>vz</td>
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<td>Tau</td>
<td>vz</td>
<td>0.62 ± 0.25</td>
<td>2.03 ± 1.25*</td>
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<td>GABA</td>
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<td>0.63 ± 0.21</td>
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$\beta$-alanine and glutamate did not differ significantly in the two supernatants. Numbers represent the mean ± SEM from three separate trials.

The contralateral slice cultured in saclofen has few BrdU+ cells in the cp (arrows). Dashed line delineates the border between the cortical plate (cp) and the intermediate zone (iz). Bar = 40 µm. (C,D) Histograms depicting the anatomical distribution of BrdU+ cells in cortical slices maintained in culture for 48h or 6 days following the BrdU pulse. (C) At 48h, nearly 80% of the BrdU+ cells are in the cortical plate (cp) of the control slice. The majority of cells in the contralateral slice exposed to saclofen are found in immature cortical regions. Over 60% of the BrdU+ cells are in the intermediate zone (iz); 22% of the BrdU-labeled cells remain in the ventricular zone (vz). (D) After 6 days of culture, virtually all BrdU+ cells are in the cp of the control slice. In the contralateral saclofen-treated slice, 70% of the BrdU+ cells have migrated into the cp, while 30% of the labeled cells remain in the iz. Numbers represent the mean from five separate trials.

**Figure 4.** Saclofen inhibits cells in immature cortical regions from migrating into the cortical plate in cultured slices. (A,B) Videomicrographs of E18 cortical slices maintained in vitro for 48h in either control medium (A) or in medium containing 100 µM saclofen (B) following an 18h BrdU pulse (see Materials and Methods). (A) The slice maintained in control medium has many BrdU+ cells in the cortical plate (arrows). (B) The contralateral slice cultured in saclofen has few BrdU+ cells in the cp (arrow). Dashed line delineates the border between the cortical plate (cp) and the intermediate zone (iz). Bar = 40µm. (C,D) Histograms depicting the anatomical distribution of BrdU+ cells in cortical slices maintained in culture for 48h or 6 days following the BrdU pulse. (C) At 48h, nearly 80% of the BrdU+ cells are in the cortical plate (cp) of the control slice. The majority of cells in the contralateral slice exposed to saclofen are found in immature cortical regions. Over 60% of the BrdU+ cells are in the intermediate zone (iz); 22% of the BrdU-labeled cells remain in the ventricular zone (vz). (D) After 6 days of culture, virtually all BrdU+ cells are in the cp of the control slice. In the contralateral saclofen-treated slice, 70% of the BrdU+ cells have migrated into the cp, while 30% of the labeled cells remain in the iz. Numbers represent the mean from five separate trials.
there was no significant death of the cells during the 30 min incubation period in saline (see Materials and Methods), indicating that the increased levels of amino acids observed in the supernatants resulted from amino acid release rather than from cell lysis.

Results from other HPLC analyses suggest that when cp cells are placed in the lower wells of the chemotaxis chamber, the cell density correlates with the levels of amino acids released. In a preliminary study, 90,000 or 300,000 cp cells were placed in the lower half of the chamber, allowed to incubate for 18 h, then HPLC was used to measure amino acids levels in the supernatants. In these studies, micromolar levels of amino acids were consistently detectable in the supernatants from cells containing high numbers of cells, whereas sub-micromolar levels were detected in the supernatants of wells containing low numbers of cells (unpublished observations).
amino acid can act as a chemoattractant for cells in the immature cortical regions and in the cp.

Discussion
We have shown that embryonic rat cortical cells express GABA\_R1 proteins in vivo and that the cells exhibit saclofen-sensitive migratory responses to GABA, indicating that embryonic cortical cells express functional GABA\_Rs. We demonstrated that (i) cortical plate cells release diffusible factors that stimulate vz cell migration via GABA\_R1s, (ii) cp cells release GABA and taurine, and (iii) both amino acids induce nerve cell movement via activation of GABA\_R2s. Together, these findings suggest that during cortical morphogenesis, cp cells release GABA and/or taurine, which act as chemoattractants to direct neuronal migration towards their target destinations in the cortical plate via GABA\_R1 signaling.

Embryonic Cortical Cells Express GABA\_R Receptors
GABA\_R1s are heteromeric receptors comprised of GABA\_R1 and GABA\_R2 receptor subunits. Physiological responses to GABA appear to require both classes of subunit proteins (Jones et al., 1998; Kaupmann et al., 1998b; White et al., 1998; Kuner et al., 1999). Here, we demonstrated the presence of GABA\_R1 subunits in the embryonic cortex. Since the cells in these studies exhibited functional responses to GABA that are pharmacologically characteristic of native GABA\_R1s (Kerr and Ong, 1992), the embryonic cortical cells presumably also express GABA\_R1 receptor subunit proteins. Studies designed to identify mRNA and protein for GABA\_R2 subunits would confirm that prior to birth, cortical cells contain both classes of proteins necessary to form fully functional GABA\_R1s.

Our studies demonstrated that in vivo, embryonic cortical cells contain mRNA and protein for both the R1a and R1b isoforms of GABA\_R1s. These two subunits result from alternative splicing of the same gene, but differ structurally in their N-terminal regions (Kaupmann et al., 1997). In neurons, GABA\_R1s couple to membrane K\(^+\) and Ca\(^{2+}\) channels as well as to adenylate cyclase, by G\(_{\alpha_i}\) proteins (Kerr and Ong, 1992). Studies with pertussis toxin indicate that the toxin uncouples post-synaptic GABA\_R1s from K\(^+\) channels but is ineffective on the presynaptic receptors that down-regulate voltage-gated Ca\(^{2+}\) channels (Dutar and Nicoll, 1988; Harrison, 1990). Pertussis toxin has been shown to effectively block embryonic cortical cell migration to GABA (Behar et al., 1998), implying that the receptors that mediate motility signals in cortical cells contain subunits that are similar to the subunits that comprise post-synaptic GABA\_R1s in mature neurons.

Recent studies report that in the mature CNS, R1a and R1b isoforms of GABA\_R1s have different anatomical and subcellular distributions (Kaupmann et al., 1998a; Benke et al., 1999; Bischoff et al., 1999). In the mature brain, the R1b isoform is more prevalent than the R1a isoform (Benke et al., 1999). However at birth, the ratio is reversed; the R1a subunit is the most abundant isoform in the developing brain (Fritsche et al., 1999). These findings indicate that the R1a subunit is transiently expressed at high levels in the developing CNS. Such a transient pattern of expression suggests that receptors containing the R1a subunits have a role in developmental processes.

The expression of functional GABA\_R1s by fetal nerve cells has been previously reported in other brain regions. Obrietan and van den Pol presented evidence that embryonic hypothalamic neurons express functional GABA\_R1 receptors (Obrietan and van den Pol, 1998). The authors reported that hypothalamic neurons taken at E15 and cultured for 3 days exhibited GABA\_R1 responses in both cell bodies and neurites (Obrietan and van den Pol, 1998). In our studies, GABA\_R1 immunoreactivity was also evident in cortical cell bodies and neurites, suggesting that this subcellular receptor distribution may be typical for GABA\_R1 neurons in the developing brain. In their studies, GABA\_R1 activation resulted in a modulation of the rise in cytosolic calcium (\([\text{Ca}^{2+}]_c\)) that embryonic neurons typically exhibit when GABA\_R1 receptors are activated. Thus, they postulated that the GABA\_R1 receptors serve to modulate \([\text{Ca}^{2+}]_c\) concentrations in embryonic cells. In the developing cortex, GABA\_R1 receptors may have a similar function, moderating \([\text{Ca}^{2+}]_c\) to levels that are permissive for the cytoskeletal rearrangements necessary for cell movement.

Modulation of \([\text{Ca}^{2+}]_c\) levels may regulate the rate of neuronal migration in the developing cortex. The results of the slice culture studies indicated that GABA\_R1 blockade slowed but did not arrest the migration of vz cells into the cp. Amino acids have been shown to regulate the rate of neuronal motility through Ca\(^{2+}\)-dependent mechanisms in the developing cerebellum. Komuro and Rakic reported that glutamate influenced the rate of granule cell migration in postnatal cerebellar slices by modulating levels of \([\text{Ca}^{2+}]_c\) (Komuro and Rakic, 1993).

Taurine as a Chemoattractant
Several lines of study provide evidence that cp cells release factors that signal postmitotic neurons in germinal regions to migrate. In the slice cultures, an exogenous attractant was not applied to the culture medium, yet blockade of GABA\_R1s with saclofen resulted in an inhibition of postmitotic cell migration. This finding suggests that the cortical cells in situ released factors that stimulated the postmitotic cells in the immature cortical regions to migrate. In the in vitro chemotaxis assay, acutely dissociated cp cells released a diffusible factor that stimulated the vz cells to migrate. The HPLC studies revealed that cp cells release several amino acids including GABA and taurine. Previous studies have reported that immature neurons release GABA and taurine from growth cones. Taylor et al. showed that growth cones from immature neurons lacking synaptic vesicle proteins released GABA and taurine in a Ca\(^{2+}\)-independent manner (Taylor et al., 1990). Together, these findings suggest that in vivo, cortical plate cells may release these two amino acids. Amino acids released into the interstitial space could diffuse towards the postmitotic cells in the immature cortical regions, establishing concentration gradients that signal the postmitotic neurons to begin their migration towards their target destinations in the superficial cortical regions.

Taurine has long been thought to influence nervous system development. For decades it has been known that taurine is the free amino acid present in the highest concentrations in newborn and neonatal brain, where its levels are three to four times greater than in the mature brain (Sturman, 1988). Taurine levels are particularly high in embryonic and newborn cortical regions and cerebellum, where concentrations of taurine peak prior to and during the period of synapse formation; taurine levels decrease between birth and weaning (Sturman, 1988). The transient expression of high taurine levels implies that the amino acid may be a trophic or trophic factor during brain development.

Studies of the brains of offspring from taurine-deficient mothers have provided evidence that the amino acid is important for neuronal migration. Examination of the cerebellum in 8-week-old kittens born to taurine-deficient mothers revealed a persistence of cells in the cerebellar external granule layer.
indicating a granule cell migration failure occurred in these animals (Sturman et al., 1985). In the developing cortex, taurine also appears to influence neuronal migration. The visual cortices of newborn kittens born to taurine-deficient mothers showed abnormalities associated with migration and differentiation. In these animals, neuroblasts were aggregated in ventricular and pial zones, indicating a migration failure had occurred during corticogenesis (Palckal et al., 1986). These studies provide direct evidence that taurine influences neuronal migration in vivo. The mechanisms through which taurine affects cell migration are unknown; however, the results of the present study suggest that in vivo, taurine may play a role as a chemoattractant, acting as a ligand at GABAB-Rs in the developing CNS. Our studies demonstrate that exogenous taurine can act as a chemoattractant for embryonic cortical cells during cortical morphogenesis in the mouse (Fritschy et al., 1999).

An examination of the brains of transgenic mice with a knockout of both isoforms of glutamic acid decarboxylase (GAD), the enzyme that synthesizes GABA from glutamate, revealed cortices that appeared morphologically normal, suggesting that GABA may be involved in directing nerve cell movement (Ji et al., 1999). These findings contrast with earlier studies demonstrating a chemotactic effect of GABA in the developing cortices of rats (Behar et al., 1996, 1998). We demonstrated, however, that in mice, glutamate rather than GABA is a primary chemoattractant during late stages of cortical development (Behar et al., 1999). The results of the current study on rats shows that taurine is released from cp cells and induced vz cell migration, raising the possibility that chemotropic effects of taurine may have contributed to cortical morphogenesis in the mouse mutants. During development, taurine may normally function as an attractant for embryonic cortical neurons in rats and mice, stimulating cell migration by GABAB-R activation.

In the present set of studies, two assays of cell migration were used. In one assay, embryonic cortical cells were enzymatically dissociated into single-cell suspensions, exposed to attractants for 18 h, then their movement through pores of a membrane were evaluated. Immunocytochemistry indicated that at the time of dissociation, ∼5–10% of the vz cells placed opposite wells containing attractants expressed GABAB-R proteins. The effect of dissociation on the vz cells’ continued capacity to proliferate and/or differentiate in vitro are unknown. In contrast, cells maintained in cortical slice cultures continue to proliferate, migrate and differentiate in vitro (Behar et al., 2000). In the slices, continuous ‘waves’ of postmitotic neurons expressing GABAB-Rs may develop within the vz over several days. Thus, by the end of the slice culture assay, numerous GABAB-R+ cells capable of responding to chemotropic signaling may be generated. When examined at a single time point, only a small percentage of vz cells may express immunocytochemically detectable GABAB-Rs, however, over a period of several days, application of saclofen would inhibit the movement of a large number of GABAB-R+ cells generated throughout the culture period.

The present studies suggested that there was an enrichment of GABAB-R+ cells in the dissociated population that migrated to GABA. Alternatively, the apparent enrichment could reflect the emergence of GABAB-R+ cells following an 18 h incubation period. However, blockade of GABA-Rs clearly attenuated cortical cell migration in both the dissociates and the slices. These findings provide strong support that signaling through GABAB-Rs stimulates neuronal movement in the developing cortex. In vivo, the natural ligand(s) that induce neuronal migration remains to be resolved, however, the results of the present study implicate at least two amino acids, GABA and taurine, as candidate attractants in the rat. Both molecules are highly expressed near the target destinations for migrating neurons, both molecules are released by cp cells, and both molecules stimulate cell movement by mechanisms involving GABAB-Rs. Together these findings suggest that in vivo, taurine and GABA act as chemoattractants for embryonic neurons during cortical histogenesis.

References

Notes
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