Blockade of $GABA_B$ Receptors Alters the Tangential Migration of Cortical Neurons

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To better understand the role of neurotransmitter receptors in neuronal differentiation and maturation a detailed knowledge of their identity, location and function in the plasma membrane of specific neuronal populations during development is required. Combining pre-embedding immunocytochemistry with cell tracking in embryonic brain slice cultures we show that virtually all neurons (~98%) migrating through the lower intermediate zone (LIZ) on their way from the medial ganglionic eminence to the cerebral cortex, express $GABA_BR1$. Blockade of $GABA_B$Rs with a specific antagonist, CGP52432, resulted in a concentration-dependent accumulation of these tangentially migrating neurons in the ventricular/subventricular zones (VZ/SVZ) of the cortex and fewer cells were observed in the cortical plate/marginal zone (CP/MZ) and LIZ. Moreover, they had significantly shorter leading processes compared with similar migrating cells in control slices. Electrophysiological recording in LIZ and CP cells revealed no direct effect of either CGP52432 or the $GABA_B$R agonist, baclofen, on resting membrane properties suggesting that the effect of CGP52432 on migration might be mediated through a metabotropic action or the regulation of release of factors controlling migration. These results suggest that $GABA_B$Rs have an important modulatory role in the migration of cortical interneurons.

Introduction

The two main neuronal types of the mammalian cerebral cortex, excitatory pyramidal cells and inhibitory non-pyramidal cells, are generated in distinct proliferative zones. Pyramidal neurons arise from the germinal ventricular zone (VZ) and migrate along the processes of radial glia to take up their positions in the developing cortex (Rakic, 1990). Non-pyramidal cells, a diverse group of mostly γ-aminobutyric acid (GABA)-containing interneurons displaying a range of morphologies and molecular identities, originate predominantly in the medial ganglionic eminence (MGE) of the ventral telencephalon (Lavdas *et al.*, 1999; Sussel *et al.*, 1999; Wichterle *et al.*, 1999; Parnavelas, 2000; Marín and Rubenstein, 2001) and follow tangential migratory routes through the intermediate zone (IZ) to reach the cortex (De Carlos *et al.*, 1996; Anderson *et al.*, 1997a,b, 2001; Tamamaki *et al.*, 1997; Letinic *et al.*, 2002). A recent study has demonstrated that the caudal ganglionic eminence is also a source of local-circuit interneurons in addition to inhibitory projection neurons and glia (Nery *et al.*, 2002).

In the mature CNS, physiological actions of GABA are mediated via activation of both ionotropic and metabotropic receptors (Macdonald and Olsen, 1994; Misgeld *et al.*, 1995; Johnston, 1996). Ionotropic GABA receptors ($GABA_A$Rs and $GABA_C$Rs) form Cl^- channels (Bormann, 1988; Sivilotti and Nistri, 1991; Feigenspan *et al.*, 1993), whereas metabotropic GABA receptors ($GABA_B$Rs) couple to Ca^{2+} and K^+ channels or adenylate cyclase via Gi/o GTP binding proteins (Kerr and Ong, 1992). Functional $GABA_B$Rs are heterodimers and so far two receptor subunits, $GABA_B$R1 and $GABA_B$R2, have been identified (Misgeld *et al.*, 1995; Kaupmann *et al.*, 1997, 1998; Bowery, 1997).

During rat cortical development, GABA is released from cells located close to the target destinations for migrating neurons and it has been shown that embryonic cortical neurons exhibit migratory responses to this neurotransmitter *in vitro* (Lauder *et al.*, 1986; Behar *et al.*, 1996, 1998, 1999, 2001; Maric *et al.*, 2001). These findings suggest that GABA is a chemoattractant during corticogenesis.

Information concerning the role of $GABA_B$Rs in the developing cerebral cortex is still sparse; however, several lines of evidence suggest that their activation might play an important role in cortical development. First, functional $GABA_B$Rs have been identified in the developing cerebral cortex (Janigro and Schwartzkroin, 1988; Cherubini *et al.*, 1991; Gaiarsa *et al.*, 1991; Fukuda *et al.*, 1993). Second, pharmacological studies indicate that $GABA_B$R activation stimulates migration of neurons in immature cortical regions (Behar *et al.*, 1998, 2000, 2001). Third, recent experiments using an *in vitro* chemotaxis assay demonstrated that cortical plate (CP) cells release GABA and taurine, both of which act as a chemoattractant for immature cortical neurons, causing the cells to migrate from the VZ into the CP via activation of $GABA_B$Rs (Behar *et al.*, 2001). In addition, recent studies have investigated the expression and distribution of $GABA_B$Rs during early cortical development (Behar *et al.*, 2001; López-Bendito *et al.*, 2002), and demonstrated a particularly high density of the receptor in tangentially orientated neurons in the lower intermediate zone (LIZ) of the cortex (López-Bendito *et al.*, 2002).

To investigate whether the activation or blockade of $GABA_B$Rs can modulate the tangential migration of cortical interneurons we have used *in vitro* embryonic organotypic cultures, in combination with immunohistochemical techniques. We have also used whole-cell and perforated patch-clamp recordings to analyze the electrophysiological properties of the migratory cells as well as the functionality of their $GABA_B$Rs. Our findings indicate that blockade of $GABA_B$Rs modifies the distribution of tangential migratory cells as well the length of their leading process. The lack of a consistent direct effect of baclofen or CGP52432 on the resting membrane potential and input resistance in LIZ and CP cells raises the possibility that the effect on migration by CGP52432 seen in tangentially migratory interneurons could be indirect via an effect on neighboring cells or mediated through intracellular messenger systems and/or the regulation of release of factors controlling migration. These findings provide strong support that signaling through $GABA_B$Rs modulates neuronal migration in the developing cortex.

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Materials and Methods

Thirty-three pregnant Wistar rats, obtained from the Animal House Facilities of the Department of Human Anatomy and Genetics in Oxford, were used in the present study. The care and handling of the animals prior to and during the experimental procedures followed European Union and UK Home Office regulations, and were approved and supervised by the Animal Care and Use Committee of the institution. We have endeavored to minimize both the suffering and the number of animals used in this study.

Organotypic Cultures

Pregnant rats at embryonic day (E) 15 stage of gestation (n = 33) were killed by cervical dislocation. Fetuses were collected by cesarean section of the dam. The fetuses (n = 91) were rapidly removed and placed in artificial cerebrospinal fluid (ACSF), in which NaCl was substituted with equiosmolar sucrose, at 4°C. The composition of the ACSF was (in mM): KCl, 3; $NaHCO_3$, 24; NaH_2PO_4, 1.25; $MgSO_4$, 2; $CaCl_2$, 2; D-glucose 10; and sucrose, 252. The brains were dissected from the skull and embedded in a 3% solution of type VII low-gelling agarose (Sigma) in Gey's balanced salt solution (GBSS, Gibco), pH 7.4, at 40°C. Brains were then cut coronally at 300 μm with a vibrating blade microtome (Leica VT1000S, Nussloch, Germany). Slices containing the MGE and primordial cortex were placed onto Millicell 0.45 μm, black gridded, 13 mm membranes (Millipore) in 30 mm Petri dishes containing 250 μl of DMEM/F12 (Life Technologies) with 2.4 mg/ml D-glucose, 5 μl/ml N_2 supplement (Gibco), 0.1 mM glutamine, 5 units/ml penicillin, 5 μg/ml streptomycin, and 10% fetal calf serum (FCS, Gibco) for 1 h at 37°C with 5% CO_2. The procedures described above were performed under sterile conditions.

Injection of Fluorescent Tracer

To examine whether the tangentially migratory neurons express $GABA_BRs$ during their migration, the MGE (n = 220) of the two hemispheres derived from E15 brains was injected with 0.7 μm tungsten-M10 particles (Bio-Rad) impregnated with 1 mM 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (Cell-Tracker Orange CMTMR; Molecular Probes) using a glass micropipette. These labeled cultures provided a reliable *in vitro* assay to test the effect of agonist or antagonist at $GABA_BRs$.

Drugs and Control

To determine the role of $GABA_BRs$ in tangential migration, we used a specific agonist (RS)-Baclofen ((RS)-4-amino-3-(4-chlorophenyl)butanoic acid; Tocris) or antagonist CGP52432 (3-[[[(3,4-dichlorophenyl)methyl]-amino]propyl] diethoxymethyl)phosphinic acid; Tocris) at $GABA_BRs$ that was added to the culture media 1 h after placement of the CMTMR. Three experimental groups were used: (i) control group grown in culture medium alone; (ii) agonist group, 100 μM of the specific $GABA_BR$ agonist, baclofen was added to the culture medium; and (iii) antagonist group, different concentrations (10 nM, 50 nM, 200 nM, 1 μM, 5 μM or 60 μM) of the selective $GABA_BR$ antagonist CGP52432 were added to the culture medium. Slices from the three experimental groups were cultured at 37°C in a mixture of 5% CO_2 and 95% air for 24 h. Slices were then fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 2 h at 4°C, and subsequently rinsed in PBS, coverslipped in PBS/glycerol solution (1:1), and observed with a fluorescent microscope (Leica DMR) or a laser-scanning confocal microscope (Leica TCS SP1).

Quantitative Studies

To establish the relative frequency of CMTMR-containing cells in control conditions and after $GABA_BR$ agonist or antagonist treatment, we carried out a quantitative analysis in different embryonic cortical compartments. First, in order to anatomically define those compartments, we counter-stained freshly fixed slices with the chromatin stain bis-benzimide (10 min in 2.5 μg/ml solution in 0.1 M phosphate buffer; Sigma). The use of the bis-benzimide allowed us to distinguish embryonic cortical compartments that we subsequently analyzed in three groups: MZ/CP, LIZ and SVZ/VZ. Neuronal counts and immunohistochemical experiments were carried out on those three defined cortical compartments (see Fig. 3*G–I*).

For quantitative assays, samples were taken from cultures prepared from 58 embryos using between 6 and 20 different cultures for each condition. Quantification was carried out under fluorescence microscope from slices chosen in a systematic manner from each animal and experiment. Randomly selected fields, using a 250 μm^2 reticule under a 40× objective lens, were then photographed from the three cortical compartments with a Leica DC500 digital camera. A minimum of 10 fields were chosen in each of 6–10 separate experiments. The total number of CMTMR-containing cells identified in each of the three selected cortical compartment under the different treatments was counted. Since the distributions of the different samples for each treatment did not differ from each other (Kolmogorov–Smirnov test, $P > 0.63$), the data were pooled. These data were converted to percentage of CMTMR-containing cells in each embryonic cortical compartment.

For the estimation of possible morphological changes in the CMTMR positive cells after application of $GABA_BR$ agonist or antagonist, the length of the leading processes of 90 CMTMR-containing cells identified in each embryonic cortical compartment was measured. The leading processes were measured in control conditions and in slices subjected to $GABA_BR$ agonist and antagonist treatment using a digitizing table and appropriate software (SigmaScan Pro, SPSS Inc., Chicago, IL). Measurements were carried out under fluorescence microscope from slices chosen in a systematic manner from three animals. Fifteen randomly selected fields from the three layers were then photographed with a Leica DC500 digital camera. The data were expressed as the mean length ± standard deviation (SD) of the leading processes in each embryonic cortical layer.

Statistical Analyses

The Kolmogorov–Smirnov non-parametric test was used to examine whether samples taken for each animal and experiment were from a homogeneous population. All remaining statistical comparisons were performed using Student's t-test. The differences were considered significant at the level of $P < 0.05$. Data are presented as mean ± SD, unless otherwise indicated. The dose–response data were analyzed by using a Hill equation to fit a sigmoidal function through the data points, using the built-in iterative Levenberg-Marquardt algorithm in Igor Pro (Wavemetrics, Lake Oswego, OR).

Immunohistochemical Procedures

To further characterize the expression of $GABA_BRs$ in CMTMR-labeled cells, following organotypic culturing and injection of CMTMR in the MGE, 20 slices originating from 10 fetal brains were used for immunohistochemical analysis.

Antibodies and Controls

An affinity-purified polyclonal antibody against $GABA_BR1a/b$ was raised in rabbits. The characteristics and specificity of this antibody have been described elsewhere (Kulik *et al.*, 2002).

To test method specificity in the procedures for light microscopy, the primary antibody was omitted or replaced by 5% (v/v) normal serum of the species of the primary antibody. Under these conditions no selective labeling was observed. Some sections were incubated with the primary antibody and without secondary antibodies to test for cross-reactivity of the secondary antibody and the CMTMR. No cross-labeling was detected.

Immunohistochemistry for CMTMR Cells Expressing $GABA_BR1$

Selected slice cultures that were previously analyzed and photographed with a fluorescence microscope were removed from the slides, embedded in 4% agarose and subsequently re-sectioned at 60 μm with a microtome (Leica VT1000S). Sections were incubated in 50 mM tris-buffered saline (TBS) containing 0.1% Triton X-100 and 10% Normal Goat Serum (NGS) at room temperature for 1 h, and they were then incubated overnight at 4°C with an affinity-purified polyclonal antibody against $GABA_BR1a/b$, at a final protein concentration of 1–2 μg/ml, in TBS containing 1% NGS. After washing in TBS, sections were incubated with Cy2-conjugated goat anti-rabbit antibody diluted 1:200 in TBS for 2 h. Immunolabeled sections were mounted in PBS/glycerol and observed using a laser-scanning confocal microscope (Leica TCS SP1). Images were stored and analyzed using appropriate software supplied by the microscope manufacturer (Leica). Brightness and contrast were adjusted for the whole frame using appropriate software.

Immunohistochemistry for $GABA_BRs$ Using the Immunoperoxidase Method

Fetuses (n = 10) at E16 were collected by cesarean section after anesthesia of the dam with an intraperitoneal injection of a Rompun/Imalgene mixture (0.1 ml/kg BW). Animals were deeply anesthetized by hypothermia and then perfused with 4% PFA in PBS thought the heart (López-Bendito *et al*., 2001, 2002). After perfusion, tissue blocks containing the neocortex were dissected and washed thoroughly in 0.1 M phosphate buffer (PB, pH 7.4). Coronal 60 μm sections were cut with a microtome (Leica VT1000S) and collected in 0.1 M PB. For the immunoperoxidase method, sections were incubated in 10% normal goat serum (NGS) in 50 mM Tris buffer (pH 7.4) containing 0.9% NaCl (TBS), with 0.2% Triton X-100, for 1 h. Sections were then incubated at 4°C for 48 h with affinity-purified polyclonal antibodies against $GABA_BR1a/b$ at a final protein concentration of 1–2 μg/ml in TBS containing 1% NGS. After several washes in TBS, the sections were incubated at room temperature for 2 h in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:50 in TBS containing 1% NGS. They were then transferred into avidin–biotin–peroxidase complex (ABC kit, Vector Laboratories) diluted 1:100 and left for 2 h at room temperature. Peroxidase enzyme activity was revealed using 3,3′-diaminobenzidine tetrahydrochloride (DAB; 0.05% in TB, pH 7.4) as the chromogen and 0.01% H_2O_2 as the substrate. Finally, the sections were air-dried and coverslipped prior to observation in a Leica DMRS photomicroscope equipped with differential interference contrast optics.

Electrophysiology for $GABA_BRs$ in Embryonic Organotypic Culture Slices

Intracellular Recordings and Data Analysis

Patch-clamp recordings were obtained using conventional procedures (Sakmann and Stuart, 1995). Briefly, brain slices (300 μm) were prepared from E16 embryos (n = 72) as described above for organotypic cultures, and were maintained for at least 1 h in culture medium (equilibrated with 5% CO_2 and 95% O_2) at 37°C before recording commenced. They were then transferred one by one to a standard submerged-style recording chamber and superfused at a rate of 2–3 ml/min with ACSF of the following composition (in mM): NaCl, 126; KCl, 3; NaH_2PO_4, 1.25; $MgSO_4$, 2, $CaCl_2$, 2; $NaHCO_3$, 26; and D-glucose, 10. All recordings were made at room temperature (22–25°C). Cells were identified using an upright fixed-stage microscope (Zeiss Axioskop, Jena, Germany) fitted with differential interference contrast optics and an infrared-sensitive video camera (Newvicon; Hamamatsu, Tokyo, Japan).

Current-clamp recordings using whole-cell and perforated patch configurations were obtained with an Axoclamp-2B amplifier (Axon Instruments, Union City, CA) in bridge mode. Recording pipettes with fine tips (pipette resistance, 8–15 MΩ for whole-cell recording; 6–7 MΩ for perforated patch-clamp recording) were pulled from borosilicate glass tubing. The pipette solution (pH, 7.35; 285–295 mOsm) contained (in mM): K gluconate, 130; HEPES, 40; NaCl, 4; MgATP, 4; and GTP, 0.3; with addition of biocytin, 5 mg/ml. pH was adjusted with KOH. For perforated patch recording, the tip was filled with pipette solution as above, omitting biocytin, and backfilled with solution of the same composition supplemented with 25 μg/ml gramicidin (Sigma), in 0.1% DMSO. Typical final access resistances were 30–70 MΩ (whole-cell recording) and 50–100 MΩ (perforated patch recording). Junction potentials were not corrected. Capacitance compensation was maximal and bridge balance was monitored and adjusted as required. The voltage responses to negative and positive current steps were recorded, filtered at 1 kHz, digitized at 2 kHz, stored on computer on-line (ITC-16, Instrutech, Long Island, NY), and subsequently analyzed off-line using custom-made procedures in Igor Pro (WaveMetrics, Lake Oswego, OR). To test the electrophysiological properties of $GABA_BRs$ in tangentially migratory and CP cells, the following compounds were added to ACSF during the recording: 300 μM (RS)-Baclofen (Tocris) and 5 μM CGP 52 432 (Tocris). All results are given as mean ± SD. Statistical significance was assessed using a two-tailed unpaired Student's t-test ($P < 0.05$). After recording, the slices were fixed in 4% PFA in PBS for 4 h. Subsequently, the slices were incubated in Cy2-conjugated streptavidin diluted 1:500 in 0.3% Triton X-100 in TBS for 3 h. Finally, the sections were mounted in PBS/glycerol and observed using a laser-scanning confocal microscope (Leica TCS SP1).

Results

$GABA_BRs$ Are Expressed in the Tangentially Migratory Neurons

We have previously demonstrated with immunohistochemical methods that $GABA_BR1$ is present in cells oriented tangentially along the LIZ of the neocortex, suggesting that this receptor is expressed in the migrating neurons presumably originating from the MGE (López-Bendito *et al*., 2002). To test this hypothesis we conducted experiments in organotypic cultures prepared from E15 rat brain. We labeled the tangentially migratory cells by placing a small number of tungsten particles impregnated with the cell tracker CMTMR in the MGE (Alifragis *et al*., 2002), and then performed immunohistochemical experiments for $GABA_BRs$ on the same cultured slices.

Most of the CMTMR-labeled cells in the LIZ were bipolar with tangentially oriented processes (Figs 1 and 2). Their morphology was very similar to that found in previous tracing and immunocytochemical studies on tangentially migrating cells (O'Rourke *et al*., 1995; Tamamaki *et al*., 1997; Lavdas *et al*., 1999). Most of these labeled neurons possessed a long leading process, which in some cases was branched.

Immunoreactivity for $GABA_BR1$ observed after 24 h in cultured re-sectioned material (Figs 1*C*,*D* and 2) was indistinguishable from that observed in freshly fixed E16 slices labeled using a pre-embedding immunoperoxidase method (Fig. 1*A*,*B*). At E16, we found strong immunoreactivity for $GABA_BR1$ in the neocortical primordium. The cortical plate (CP), marginal zone (MZ) and subplate (SP) were immunoreactive for $GABA_BR1$ (Fig. 1*A*,*B*). In addition, $GABA_BR1$ immunoreactivity was also present in the tangentially migratory cells in the LIZ (Fig. 1*A*,*B*). In the re-sectioned material, the receptor was similarly localized at the plasma membrane and in the cytoplasm of cells located in the LIZ, MZ, CP and SP (Figs 1*C*,*D* and 2*I*,*K*). The CMTMR labeling was observed mainly in the nucleus and cytoplasm (Fig. 2*J*,*K*) of cells located in the LIZ with tangential orientation. Virtually all CMTMR-labeled cells, ~98% (n = 260 CMTMR-labeled cells examined in n = 4 cultures of E15 rats), expressed the $GABA_BR1$ subunit (Figs 1*D* and 2*F*,*G*,*H*,*K*). This result indicates that $GABA_BRs$ are expressed in migrating cells originating in subpallial areas.

Blockade of $GABA_B$ Receptors Changes the Distribution of Migrating Neurons in Organotypic Cultures

Recent pharmacological studies have provided evidence that activation of GABA receptors by endogenous GABA can modify cortical neuronal migration (Behar *et al*., 2000, 2001). To test whether $GABA_BRs$ play a role in the migration of the tangentially migratory neurons, we investigated the effect of the application of a specific antagonist at $GABA_BRs$, CGP52432, in organotypic cortical slice cultures. We placed tungsten particles impregnated with 1 mM CMTMR in the MGE (Fig. 3*A*–*C*), the main source of cortical tangentially migratory GABAergic neurons (Anderson *et al*., 1997a; Lavdas *et al*., 1999), in E15 rat brain slices to label that specific neuronal population. We incubated the slices for 24 h under three conditions: (i) standard culture medium, (ii) medium with the addition of 100 μM of the specific $GABA_BR$ agonist, baclofen, and (iii) medium with the addition of different concentrations of CGP52432 (10 nM, 50 nM, 200 nM, 1 μM, 5 μM or 60 μM). After incubation, the slices were fixed and counterstained with bis-benzimide to enable identification of cortical layers based on their cell density (Fig. 3*G*–*I*). In the presence of 200 nM, 1 μM, 5 μM or 60 μM CGP52432 we observed significantly more CMTMR-labeled cells located in the

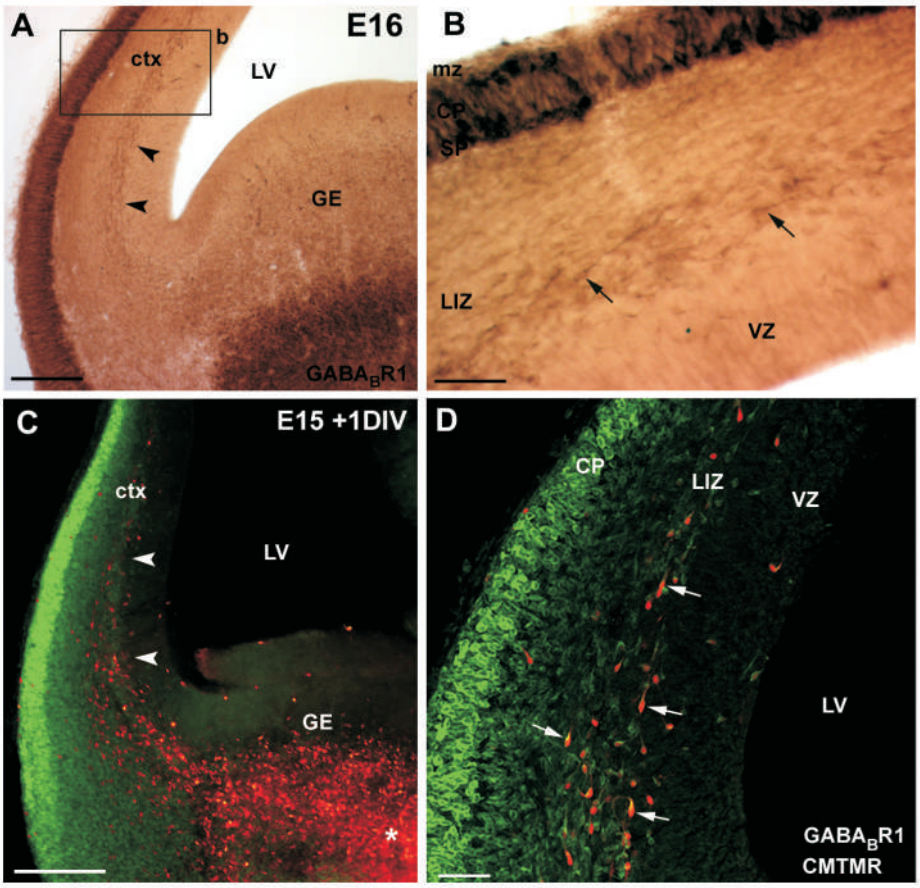


Figure 1. Immunoreactivity for GABA$_B$R1 in the rat telencephalon at embryonic day 16 (E16) using pre-embedding immunoperoxidase (*A* and *B*) and immunofluorescence methods (*C* and *D*). (*A*, *B*) Immunoreactivity for the GABA$_B$R1 subunit present in cells of the marginal zone (mz), cortical plate (CP), subplate (SP) and lower intermediate zone (LIZ; arrowheads in *A* and arrows in *B*). (*C*, *D*) E15 brains were cut at 300 μm and coated CMTMR particles were placed into the medial ganglionic eminence (MGE). After 24 h *in vitro*, the slices were fixed, re-sectioned at 60 μm and an immunofluorescence reaction for GABA$_B$R1 was performed. The CMTMR-positive cells were observed at the LIZ, CP and mz. The majority of the CMTMR labeled cells (red) co-expressed the receptor (green) (arrows). Abbreviations: ctx, cortex; LV, lateral ventricle; GE, ganglionic eminences; VZ, ventricular zone. Scale bars: *A* and *C*, 200 μm; *B* and *D*, 50 μm.

VZ/SVZ relative to baclofen and control conditions (Fig. 3*E*; Fig. 4). Moreover, after CGP52432 treatment, less CMTMR positive cells were found in the MZ/CP or LIZ in comparison with control and baclofen treatment (Fig. 4). No differences in the percentage of CMTMR cells per cortical compartment were observed between control and baclofen treatment (Fig. 4).

To investigate whether, in the presence of CGP52432, the expression of the GABA$_B$R1 subunit in the CMTMR positive cells changed, we performed immunofluorescence experiments for GABA$_B$R1 in culture sections that were incubated for 24 h with CGP52432 (Fig. 2*G*,*H*). The percentage of GABA$_B$R1 positive CMTMR-labeled cells after CGP52432 treatment did not differ from control (control, 98%; n = 257 CMTMR cells examined; CGP52432 60 μM, 98%; n = 155 CMTMR cells examined). GABA$_B$R1 expression was mainly localized in the cytoplasm and plasma membrane (Fig. 2*I*) of LIZ, MZ, CP and SP cells and no differences in immunoreactivity or localization were found under this treatment condition in comparison with control slices.

The Effect of CGP52432 Does Not Depend on Rostro-caudal Telencephalic Level

To test whether the increase of CMTMR-positive cells in the VZ/SVZ caused by CGP52432 is dependent upon any particular rostro-caudal telencephalic level we prepared serial brain slices in which pharmacological studies using the same drug conditions as described above were carried out. CMTMR-labeled cells were found accumulated in the VZ/SVZ after the CGP52432 treatment at caudal as well as at rostral brain levels (Fig. 4*A*,*B*). Fewer CMTMR-positive cells were found in the MZ/CP and LIZ in slices treated with the GABA$_B$R antagonist at both rostro-caudal levels (Fig. 4). No differences in the percentage of CMTMR-labeled cells located in different cortical compartments were observed between control and baclofen situations at rostral and caudal levels (Fig. 4).

The CGP52432 Concentration–Effect Relation Is Consistent with an Action Via GABA$_B$Rs

To investigate whether the effect of CGP52432 on tangential migration is mediated via specific antagonism at GABA$_B$R we quantified the number of CMTMR positive cells per cortical compartment using different concentrations of CGP52432. A Hill equation was used to fit a sigmoidal function through the data points. The estimated concentration of CGP52432 that gave half of the maximal effect was 31 nM for LIZ and 125 nM for SVZ/VZ (Fig. 5). This compares to the reported IC$_{50}$ of 85 nM at rat cortical GABA$_B$ autoreceptors (Lanza *et al.*, 1993). Thus, the result is consistent with an effect via blockade of GABA$_B$R.

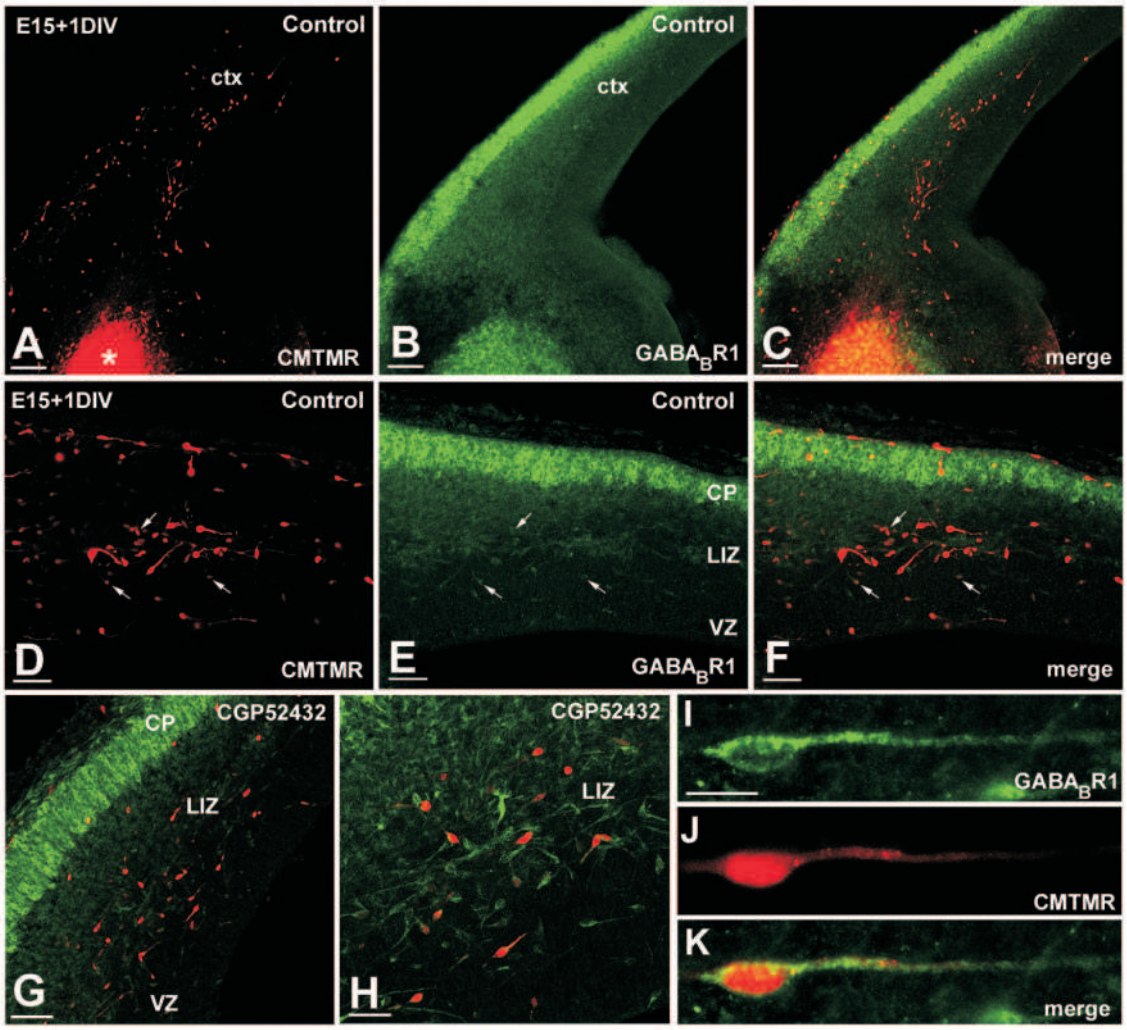


Figure 2. Distribution of CMTMR-positive cells labeled from the medial ganglionic eminence (MGE) (*A*, *C*, *D*, *F–H*, *J* and *K*) and immunoreactivity for GABA$_B$R1 using a pre-embedding immunofluorescence technique (*B*, *C*, *E–I* and *K*) in the same telencephalon at E15 + 1DIV. The majority of the CMTMR-positive cells (red) labeled from the MGE express the GABA$_B$R1 subunit (green) during migration. E15 brains were cut at 300 μm coronally under sterile conditions. CMTMR coated particles were placed in the MGE of each hemisphere to label the tangential migratory neurons. The slices were then incubated for 24 h *in vitro* under two conditions: (i) Control slices, incubated in normal culture medium (A–F) or (ii) slices treated in a medium containing a specific antagonist at GABA$_B$Rs, CGP52432 (*G–K*). After 24 h of incubation, the slices were fixed, re-sectioned at 60 μm and an immunofluorescence reaction for GABA$_B$R1 was performed. In both conditions, the percentage of co-localization of CMTMR and GABA$_B$R1 was ~98%. Abbreviations: ctx, cortex; CP, cortical plate; LIZ, lower intermediate zone; VZ, ventricular zone. Scale bars: *A–C*, 100 μm; *D–G*, 50 μm; *H*, 20 μm, *I–K*, 10 μm.

We tested whether the block by CGP52432 could be overcome by an agonist by adding baclofen (300 μM) in combination with CGP52432 (at its IC_{50} concentration, 85 nM; $n = 51$). CMTMR injections were performed at the E15 MGE as was described before. After 24 h *in vitro*, no significant differences were observed in the percentage of CMTMR-labeled cells located in different cortical compartments between cultures treated with baclofen plus CGP52432 and control cultures (control: MZ, 20.4 ± 1.3%; LIZ, 56.2 ± 1.8%; SVZ/VZ, 23.3 ± 1.5%; CGP52432 85 nM + baclofen: MZ, 19.5 ± 1.8%; LIZ, 51.3 ± 1.6%; SVZ/VZ, 29.2 ± 1.3%; $P = 0.1$). Compared to the concentration–effect curve for CGP52432, these data appear to suggest a partial reversal of the CGP52432 effect; however, this result did not reach significance and was not investigated further.

GABA$_B$R Blockade Alters Neurite Morphology in Tangential Migratory Neurons

Recent studies have demonstrated that activation of AMPA-type glutamate receptors causes retraction of neurites in migrating neurons in the LIZ (Poluch *et al.*, 2001). Since the same neuronal populations express GABA$_B$Rs, we were interested in determining whether the effect of GABA$_B$R antagonists on migration might be mediated via similar morphogenetic processes. Thus, we measured the length of the leading process of CMTMR positive cells in the three experimental conditions. After treatment with 5 μM CGP52432 of E15 rat brain slices for 1 day *in vitro* (DIV), we found a significant decrease in the length of the leading process of CMTMR positive cells (Fig. 6) in the LIZ (control, 78.3 ± 13.7 μm; CGP52432, 61.9 ± 11.8 μm; $P < 0.001$) and in the VZ (control, 78.5 ± 13.3 μm; CGP52432, 61.7 ± 10.9 μm; $P < 0.001$), but not in the MZ (control, 77.9 ± 15.3 μm; CGP52432, 75.6 ± 15.8 μm; $P = 0.65$).

Lack of Direct Effect on Passive Membrane Properties by GABA$_B$R Agonists and Antagonists

In an attempt to investigate whether the effect of the GABA$_B$R antagonist on migration in tangential neurons could be mediated via a direct effect on electrical membrane properties, we

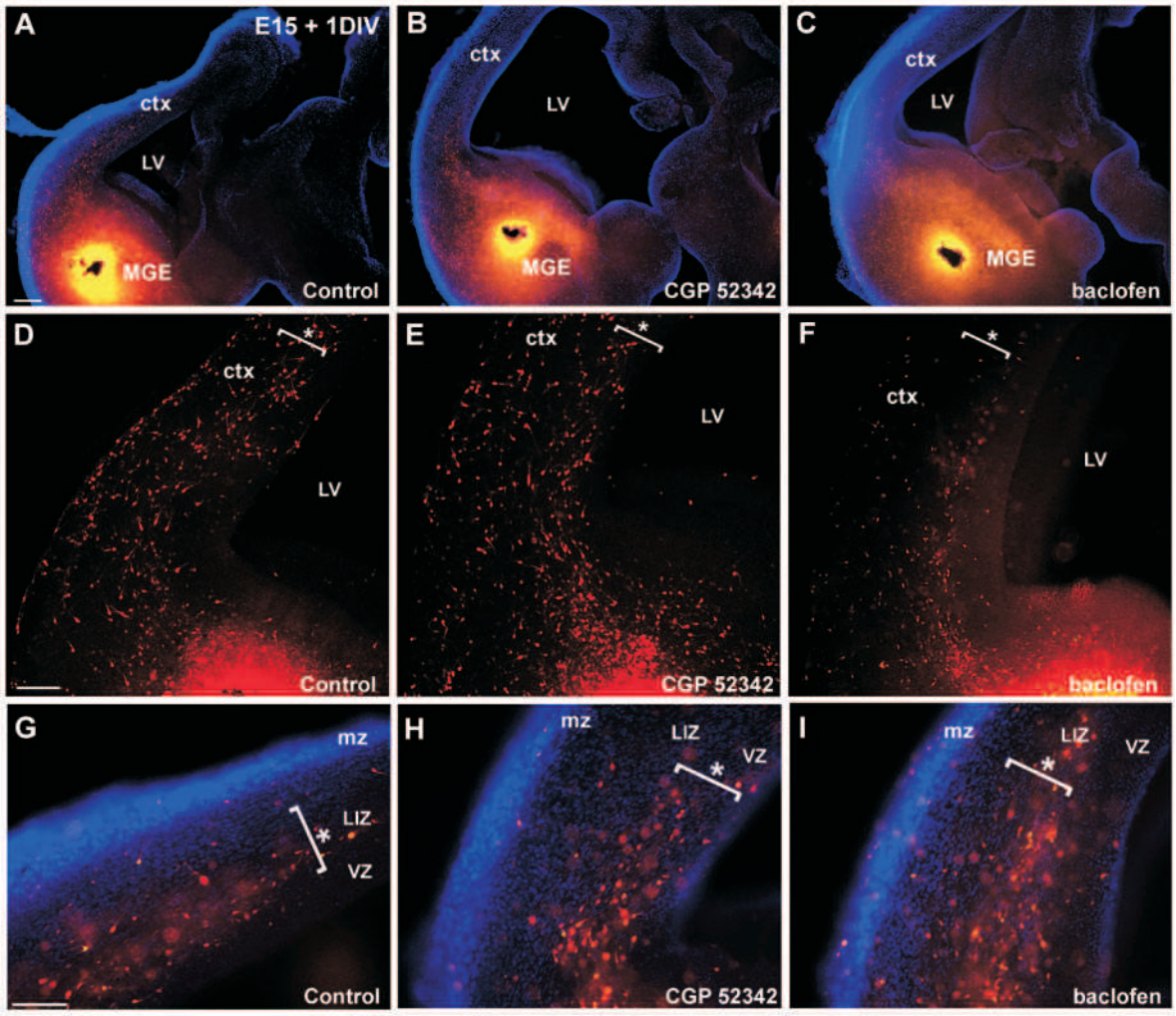


Figure 3. Effect on migration of tangentially migratory cells by specific blockade of $GABA_BRs$ with CGP52432 (*B*) and treatment with the $GABA_BR$ agonist baclofen (*C*), relative to control condition (*A*). E15 brain slices were cut at 300 μm and CMTMR-coated particles were placed in the MGE of each hemisphere to label the tangentially migratory neurons. The slices were then incubated for 24 h *in vitro* under three conditions: (i) control slices, incubated in normal culture medium (*A*, *D* and *G*), (ii) adding to the medium 1 μM of the $GABA_BR$ antagonist, CGP52432 (*B*, *E* and *H*) and (iii) adding 100 μM of the $GABA_BR$ agonist, baclofen (*C*, *F* and *I*). After the incubation period, the slices were fixed and counterstained with 0.002% bis-benzimide (blue) to reveal the different cortical compartments and brain structures. In the presence of CGP52432 more CMTMR-labeled cells located at the ventricular/subventricular zones (VZ/SVZ) were observed (*H*). Asterisks at *D–I* represent where the majority of the CMTMR-positive cells were localized. Abbreviations: ctx, cortex; LIZ, lower intermediate zone; mz, marginal zone; VZ, ventricular zone. Scale bars: *A–C*, 200 μm; *D–F*, 100 μm; *G–I*, 100 μm.

monitored the membrane potential as well as voltage response to negative and positive current steps during wash-in of either baclofen (300 μM) or CGP52432 (5 μM) in E16 rat cortical slices. No consistent effects could be seen in LIZ cells (n = 7 and 12; Fig. 7*A*i, *B*i, *C*i, *D*i, *E*). Similarly, we detected no consistent effect in CP cells at this developmental stage (n = 2 and 12; Fig. 7*A*ii, *B*ii, *C*ii, *D*ii, *E*). As a positive control, we investigated the effect of baclofen on layer V pyramidal neurons in somatosensory cortex in slices from P14 rats. In contrast to the embryonic slices, there was a robust hyperpolarization associated with a decrease in input resistance in these neurons in agreement with previous reports from neocortical pyramidal cells [n = 4; Fig. 7*A*iii, *B*iii, *C*iii (Connors *et al.*, 1988)].

One possible explanation for the lack of response to baclofen in tangential migratory cells could be rapid 'wash-out' of cytoplasmic components into the recording pipette during whole-cell recording because of the small volume of these embryonic cells. To exclude this possibility we performed perforated-patch recordings (Marty and Neher, 1995) using the antibiotic gramicidin. After wash-in of 300 μM baclofen we did not observe any significant change of the membrane potential or input resistance in LIZ cells (–49.6 ± 8.4 mV and 3.26 ± 1.14 GΩ, respectively, 10 min after wash-in of baclofen compared with –54.7 ± 8.4 mV and 3.48 ± 0.92 GΩ in control; n = 4). These results strengthen the conclusion using whole-cell patch recordings that baclofen has no direct effect on resting membrane properties in tangential migratory cells at E16.

The lack of effect of ligands at GABABRs on membrane properties of tangentially migratory neurons is consistent with previous studies, reporting no effect of baclofen on $[Ca^{2+}]_i$ in IZ migratory interneurons (Behar *et al.*, 1996; Soria and Valdeolmillos, 2002). We conclude that the effect of CGP52432 on neuronal migration is mediated via mechanisms distinct from direct changes in membrane properties of tangentially migratory neurons.

Discussion

We have reported here that exposure to CGP52432, a specific $GABA_BR$ antagonist, induced a change in the distribution of

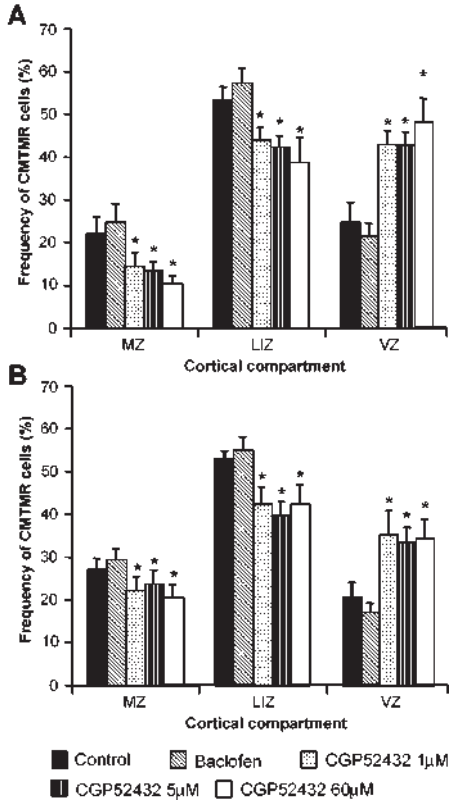


Figure 4. Effect of the $GABA_BR$ antagonist CGP52432 at 1 μM (dotted bars), 5 μM (vertically hashed bars) and 60 μM (open bars), and the agonist baclofen (diagonally hashed bars) relative to control conditions (filled bars), on the number of CMTMR-positive cells migrating through different cortical compartments. At both telencephalic levels, caudal (*A*) and rostral (*B*), the proportion of CMTMR-positive cells after treatment with the three concentrations of CGP52432 differed significantly ($*P < 0.01$) from baclofen and control conditions in the MZ, LIZ and VZ. Results are expressed as mean ± SD.

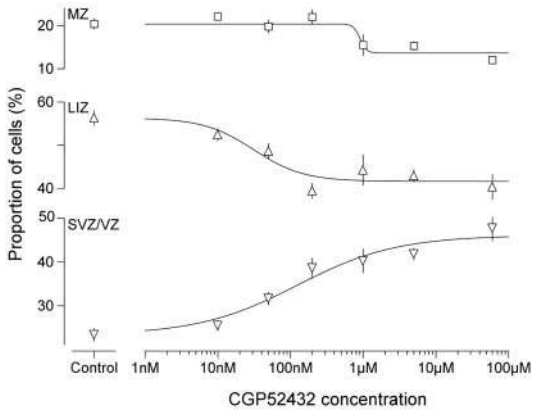


Figure 5. Concentration–effect relationship for the effect of the $GABA_BR$ antagonist CGP52432 on the tangential migration into three cortical compartments (MZ = cortical plate/marginal zone, SVZ/VZ = subventricular zone/ventricular zone, and LIZ = lower intermediate zone). A Hill equation was used to fit a sigmoidal function to each data set. The estimated concentration at half maximal effect was: MZ (squares), ~900 nM; LIZ (triangles), ~31 nM; SVZ/VZ (inverted triangles), ~125 nM. The error bars represent SEM.

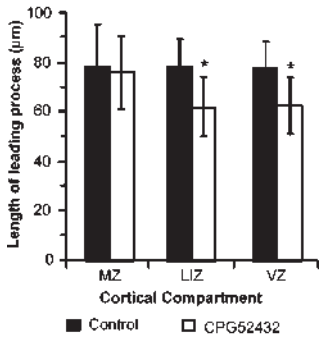


Figure 6. Length of the leading processes of CMTMR-positive cells migrating through different cortical compartments in control condition (black bars) and after treatment with the $GABA_BR$ antagonist CGP52432 (opens bars). After treatment with CGP52432 the length of the leading process was reduced significantly ($*P < 0.001$) from control conditions in the LIZ and VZ, but not in the MZ. Results are shown as mean ± SD.

tangentially migratory neurons originating in the MGE. We also showed that this drug induced a reduction of the length of their leading process. This leads us to propose that early in development, before the differentiation of synapses, $GABA_BRs$ may have a role in modulating the migration of tangentially migratory interneurons.

Tangential Interneurons Express $GABA_BRs$ during Their Migration

The expression of $GABA_BRs$ in different populations of cortical cells at very early stages of embryonic development has been described earlier (Behar *et al.*, 2001; López-Bendito *et al.*, 2002). We reported the expression of the $GABA_BR$ subunit, $GABA_BR1$, in cells located at the LIZ (López-Bendito *et al.*, 2002). In the present study, we have shown that, in fact, $GABA_BR1$ is expressed in the plasma membrane of cells migrating from the MGE towards the cortex. This was demonstrated by labeling the cells originating from the MGE with a cell-tracker, CMTMR, followed by immunostaining for the $GABA_BR1$ subunit.

Several studies have shown that migrating interneurons in the LIZ express a variety of ionotropic receptors, including AMPA-type glutamate receptors with high calcium permeability (Métin *et al.*, 2000; Poluch *et al.*, 2001) (López-Bendito, Luján and Fairén, unpublished observations), NMDA-type glutamate receptors and $GABA_A$ receptors (Soria and Valdeolmillos, 2002), the activation of which lead to changes in $[Ca^{2+}]_i$. In the present study, we demonstrate that the same cell population also expresses the metabotropic receptor $GABA_BR1$. It has been shown that GABA is capable of both stimulating and arresting the migration of embryonic cortical neurons via different signaling mechanisms and GABA-Rs (Behar *et al.*, 1998). Therefore, it might be possible that activation of $GABA_BRs$ is necessary for a proper navigation through different cortical compartments and for the acquisition of the final position in the cortical circuitry.

Blockade of $GABA_BRs$ Changes the Distribution of Tangential Migratory Cortical Interneurons

We have shown in the present study that *in vitro* application of CGP52432 in cortical embryonic slices results in a concentration dependent change in the cortical distribution of tangentially migratory neurons originating in the MGE. More CMTMR-positive cells were found in the VZ/SVZ areas of the cortex whereas fewer were found at the MZ, CP and LIZ following treatment with CGP52432. The effect was similar at rostral and caudal telencephalic levels. The concentration–effect curve was consistent with an effect via $GABA_BR$. The estimated concentration giving half of the maximal effect was close to the IC_{50} value of the drug at GABA autoreceptors in postnatal rat cerebral

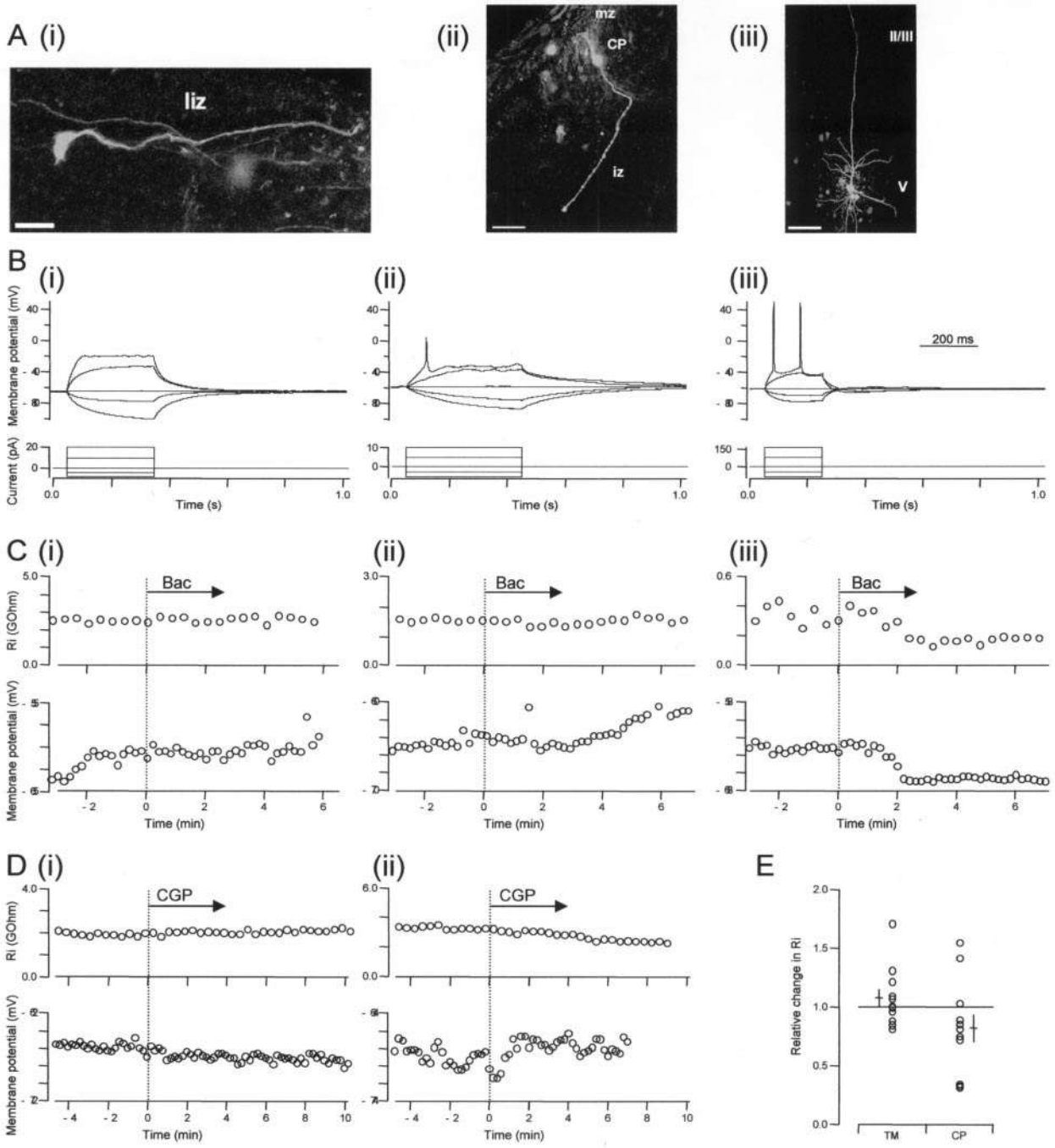


Figure 7. Response of cells in the lower intermediate zone (LIZ) at E16, cortical plate (CP) cells at E16 and layer V pyramidal cells at P14 to baclofen and CGP52432. (*A*). Morphological identification of recorded neurons. The cells were filled with biocytin during recording and their morphology revealed by Cy2-conjugated streptavidin. The examples show: a tangentially migratory neuron in the lower intermediate zone (LIZ) at E16 (i), a cortical plate (CP) cell at E16 (ii) and a layer V pyramidal neuron at P14 (iii). (i) Note the tangential disposition along the LIZ of the migratory cell. The long leading process is a well described characteristic of this type of neurons. (ii) Note also the long axon of the CP filled cell along the IZ. (*B*). Examples of responses of representative neurons to depolarizing and hyperpolarizing current steps. (i). Tangential migratory neurons failed to show action potentials. (ii). In contrast, in most CP cells a single broad action potential was elicited by depolarizing current pulses whereas all P14 pyramidal cells exhibited repetitive over-shooting action potentials (iii). (*C*). Effect of 300 μM baclofen on input resistance (upper panel) and membrane potential (lower panel) in tangentially migratory neurons (i, mean of $n = 7$ cells), cortical plate cells (ii, $n = 2$) and P14 pyramidal cells (iii, $n = 4$). The input resistance was estimated from the voltage response to small hyperpolarizing current pulses. The membrane potential was sampled twice for each estimate of input resistance. The start of drug application is indicated with a vertical dotted line. (*D*). Effect of 5 μM CGP52432 on input resistance (upper panel) and membrane potential (lower panel) in tangential migratory neurons (i, mean of $n = 12$ cells) and cortical plate cells (ii, $n = 12$). The start of drug application is indicated with a vertical dotted line. (*E*). Summary of response to CGP52432 in tangential migratory neurons and CP cells. Individual experiments are shown with open circles. The average is shown by a horizontal line with error bars representing the SEM. Scale bars: *Ai*–*Aii*, 20 μm; *Aiii*, 100 μm.

cortex (Lanza *et al.*, 1993), suggesting that the effect of CGP52432 might be mediated through the regulation of release of GABA or other factors controlling migration. Moreover, the $GABA_BR$ agonist baclofen reduced the estimated inhibitory effect of CGP52432 at IC_{50}, although this reduction did not reach significance. Altogether, these results strongly suggest that the

change in distribution of tangential migrating neurons in the cortex after CGP52432 treatment is indeed due to block of $GABA_BRs$.

Previous studies using BrdU pulses in saclofen-treated culture slices, another $GABA_BR$ antagonist, have shown an accumulation of BrdU positive cells at the level of VZ/LIZ and fewer cells were found in the CP (Behar *et al.*, 2001). The conclusion of these authors was that saclofen is able to inhibit the radial migration of postmitotic VZ cells into the CP via $GABA_BRs$ but the authors did not investigate whether tangentially migratory cells could be also affected. A ventricle-directed migration of LIZ cells has also been demonstrated (Nadarajah *et al.*, 2002). It is possible that certain migratory LIZ cells need the activation of $GABA_BRs$ to be able to 'escape' from the SVZ/VZ to enter into the CP. Therefore, the blockade of $GABA_BRs$ could produce an accumulation of those neurons at the lower cortical compartments. Because we only tested the effect of CGP52432 after 1 DIV it might be possible that the accumulation of CMTMR-labeled cells at the VZ is not a permanent effect and the cells might be only delayed from the normal timing of their entry into the CP. Long time course cultures and time-lapse studies may help resolve this question.

The failure to detect consistent changes in resting membrane properties in LIZ cells following either baclofen or CGP52432 treatment combined with the lack of detectable $GABA_BR2$ subunit of the $GABA_BRs$ (López-Bendito *et al.*, 2002), considered necessary for fully functional $GABA_BRs$ (Jones *et al.*, 1998; Kaupmann *et al.*, 1998; White *et al.*, 1998; Kuner *et al.*, 1999), raises the question of whether the effect seen might be indirect, e.g. through blockade of $GABA_BRs$ regulating release of factors controlling migration. We have previously shown that both subunits of $GABA_BRs$ are expressed in CP cells at early embryonic stages (López-Bendito *et al.*, 2002). Moreover, the presence of close appositions between elongating cortical axons and GABAergic IZ cells has been reported (Métin *et al.*, 2000). At their presynaptic location in mature neurons, $GABA_BRs$ have been shown to decrease neurotransmitter release (Bowery, 1993; Misgeld *et al.*, 1995), and thus it is conceivable that blockade of $GABA_BRs$ could enhance glutamate release, which via activation of AMPA receptors on tangential migratory neurons could arrest migration (Métin *et al.*, 2000; Poluch *et al.*, 2001).

Another possibility, however, is that even without changes in the membrane potential, $GABA_BRs$ could be mediating an effect through changes in intracellular messengers not readily detectable with electrophysiological methods, maybe involving a novel and not yet identified subunit.

Involvement of $GABA_BRs$ in Neurite Outgrowth

We demonstrated here that pharmacological $GABA_BR$ manipulation affects tangentially migratory neurons, not only at the level of the route of migration but also the length of their leading process. After CGP52432 treatment, CMTMR cells located in the LIZ and VZ had a shorter leading process compared with controls. Similar changes in the neurite length of those cells were observed after activation of AMPA receptors (Poluch *et al.*, 2001). These IZ cells express GluR1, but few if any GluR2 subunits of AMPA receptors (König *et al.*, 1998; Métin *et al.*, 2000). We do not yet know whether the reduction in the length of the leading process after $GABA_BR$ blockade is causally linked to the effect on the migration. Thus, the accumulation of migrating cells in the VZ after CGP52432 treatment might be due to the shortening of the leading process, or, to the contrary, the effect of the $GABA_BR$ antagonist on the leading process might be a consequence of the accumulation of cells at the VZ. However, the possibility that these are two independent effects cannot be ruled out at present. Further experiments are needed to solve this problem. Whatever the significance of the reduction of the length of the leading process may be, it has been proposed that if an endogenous agonist is present *in vivo* at an appropriate concentration, it is likely to increase cytosolic calcium concentration ($[Ca^{2+}]_i$) that might lead, directly or indirectly, to neurite retraction. Therefore, the possibility exists that the changes observed in neurite length after blockade of $GABA_BRs$ might be mediated indirectly through enhancement of glutamate release and subsequent activation of AMPA receptors present in the migrating neurons. Alternatively, however, a direct effect of $GABA_BRs$ through changes in cytosolic Ca^{2+} can not be ruled out, although the failure of baclofen to modulate $[Ca^{2+}]_i$ in LIZ cells makes this possibility appear less likely (Soria and Valdeolmillos, 2002).

Possible Role of $GABA_BRs$ In Vivo

Our results revealed that CGP52432 in rat embryonic cortex, most likely acting via $GABA_BRs$, alters the migration of tangential interneurons. This finding suggests that cortical cells *in situ* and maybe even the tangential migratory neurons themselves release factors that stimulate neurons to migrate in a particular manner. Our results support the idea that neurotransmitter receptors play a role in regulating migration early in development before synapses are established. A range of different molecules have been described recently to be involved in this early cortical process, such as Slit in repelling lateral ganglionic eminence neurons (Zhu *et al.*, 1999) and semaphorins 3A-3F and neurotrophins in the guidance of LIZ tangential cells from the MGE towards the cortex (Polleux *et al.*, 2002; Tamamaki *et al.*, 2003). Neurotransmitter receptors may act in concert with these guidance mechanisms.

Recently, mice lacking the $GABA_BR1$ subunit of the $GABA_BRs$ have been produced, showing behavioral, biochemical and functional alterations in the brain (Prosser *et al.*, 2001; Schuler *et al.*, 2001). Future *in vivo* and *in vitro* studies in these mice might help understand the role of $GABA_BRs$ in early stages of cortical development.

Notes

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