GABA-A Receptors Regulate Neocortical Neuronal Migration In Vitro and In Vivo

The cortical migration process depends on a number of trophic factors and on the activation of different voltage- and ligand-gated channels. We investigated the role of gamma-aminobutyric acid (GABA) type A receptors in the neuronal migration process of the newborn rat parietal cortex in vivo and in vitro. Local in vivo application of the GABA-A antagonist bicuculline methiodide (BMI) or the agonist muscimol via cortical surface Elvax implants induced prominent alterations in the cortical architecture when compared with untreated or sham-operated controls. BMI- and muscimol-treated animals revealed heterotopic cell clusters in the upper layers and a complete loss of the cortical lamination in the region underlying the Elvax implant. Immunocytochemical staining for glial fibrillary acidic protein, N-methyl-D-aspartate receptors, and GABA demonstrated that heterotopia was not provoked by glial proliferation and confirmed the presence of both glutamatergic and GABAergic neurons. In organotypic neocortical slices from embryonic day 18–19 embryos, application of BMI and to a lesser extent also muscimol induced an increase in the migration speed and an accumulation of neurons in the upper cortical layers. Spontaneous intracellular calcium ([Ca^{2+}]_i) oscillations in neocortical slices from newborn rats were abolished by BMI (5 and 20 μM) and muscimol (1 and 10 μM), indicating that both compounds interfere with [Ca^{2+}]_i signaling required for normal neuronal migration. Electrophysiological recordings from migrating neurons in newborn neocortical slices indicate that long-term application of muscimol causes a pronounced reduction (1 μM muscimol) or blockade (10 μM) in the responsiveness of postsynaptic GABA-A receptors due to a pronounced receptor desensitization. Our results indicate that modulation of GABA-A receptors by compounds acting as agonists or antagonists may profoundly influence the neuronal migration process in the developing cerebral cortex.

Keywords: GABA-A receptor, intracellular calcium, in vitro, in vivo, neuronal migration

Introduction

During early development, cortical neurons migrate from their sites of origin to their final destination where they differentiate and establish synaptic connections (for review, Sidman and Rakic 1973; Kriegstein and Noctor 2004). In vitro studies in cell culture systems or in organotypic brain slice cultures have demonstrated that the migration process is regulated by a number of different factors (for review, Komuro and Rakic 1998; Nadarajah and Parnavelas 2002; Marín and Rubenstein 2003), including gamma-aminobutyric acid A/C (GABA-A/C) (Behar and others 1996, 1998) and GABA-B receptors (Behar and others 2001; López-Bendito and others 2003). In slice cultures of embryonic rat cerebral cortex, GABA-A/C receptors are involved in the migration of neurons from the proliferative zone into the intermediate zone, whereas GABA-B receptors signal the cells to migrate into the cortical plate (Behar and others 2000).

The role of GABA and GABA receptors in modulating cortical neuronal migration in vivo is largely unknown. Using cortical implants of Elvax polymers loaded with an N-methyl-D-aspartate (NMDA) receptor antagonist, we recently demonstrated that the migration process of neocortical neurons is also regulated by NMDA receptors under in vivo conditions (Reiprich and others 2005), confirming previous in vitro studies (Komuro and Rakic 1993; Behar and others 1999; Hirai and others 1999). To examine the role of GABA-A receptors on neuronal migration in the cerebral cortex in vivo, we inserted Elvax implants containing a GABA-A receptor agonist or antagonist subdurally over the parietal somatosensory cortex of the newborn rat. Because the drug is released during the first postnatal days when neocortical neurons in rodents are still migrating, this pharmacological intervention should locally interfere with the migration process at the site of the implant. In order to study the influence of GABA-A receptor activation or blockade in more detail, we also studied the effect of bicuculline methiodide (BMI) and muscimol application on neuronal migration in vitro using organotypic neocortical slices. Because neuronal cell migration is controlled by the amplitude and frequency of intracellular calcium ([Ca^{2+}]_i) transients (for review, Rakic 1997; Komuro and Kumada 2005), we also investigated the effect of BMI and muscimol on spontaneous [Ca^{2+}]_i oscillations in the cortical plate of newborn rats. Finally, we studied with patch-clamp recording techniques the effect of prolonged GABA-A receptor activation in vitro by long-term application of muscimol. Our studies give the first proof that modulation of GABA-A receptors regulates the migration process of cerebral cortical neurons in vivo. In addition, our in vitro data indicate that drugs acting as a GABA-A receptor agonist or antagonist influence the normal migration process and may induce neuronal migration disorders.

Materials and Methods

Preparation of Elvax Implants and Drug Release

Implants were prepared as described previously (Smith and others 1995; Persico and others 1997; Reiprich and others 2005). Briefly, ethylene vinyl acetate copolymer (EVA)(40% vinyl acetate by weight, a gift from Erbslöö GmbH, Velbert, Germany) was washed for 1 week in several changes of 95% ethanol and subsequently dissolved in methylene chloride to give a 10% solution (w/v). Either BMI or muscimol (both from Sigma, Deisenhofen, Germany) was dissolved in 4% dimethyl sulfoxide (DMSO, Sigma) at a concentration of 20 mM. After adding 1% Fast Green (Sigma) to help in visualizing the Elvax slices, the suspension was vortexed for 30 min and subsequently poured onto a glass slide,
which was covered by a 2nd slide. The slides were clamped together and placed on dry ice for 30 min. A spacing of approximately 100 μm between the slides was provided by a paraffin frame (American National Can, Greenwich, CT). Elvax was stored at −20 °C for 7 days, and on the day of surgery, small Elvax slabs of 1 × 1 mm were cut under a binocular. The in vitro release of BMI and muscimol was measured by scintillation counting using implants loaded with a mixture of [3H]BMI/BMI or [3H]muscimol/muscimol, respectively, 1,500 000 labeled/ unlabeled. The implants were incubated in 500 μl Gey’s balanced salts solution at 37 °C.

The bath solution was exchanged every 24 h, and the amount of released radioactivity was measured by scintillation counting. Data concerning the EVA release kinetics were expressed in pmol/mm² for triplicate determinations.

**Animals and Surgical Implantation**

All experiments were conducted in accordance with the national laws for the use of animals in research (Deutsche Forschungsgemeinschaft 2004) and approved by the local ethical committee (#23.05-230-3-76/00). Newborn (postnatal day [P] 0; P0 was defined as the first 24 h after birth) Wistar rats were anesthetized using hypothermia. The skin overlying the parietal cortex was cut along the midline over a distance of 6–7 mm with a small scalpel. A craniotomy was made over the parietal cortex using a microdrill (Fine Science Tools, Heidelberg, Germany).

The dura was opened, and an Elvax slice containing DMSO, DMSO + BMI, or DMSO + muscimol was placed on the cortical surface in the region corresponding to the primary somatosensory cortex (Paxinos and others 1991). The cavity was covered with a piece of pericardial membrane (W. L. Gore & Associates, Inc., Flagstaff, AZ), and the skin edges were pulled together for suturing. After surgery, animals were warmed up under an infrared lamp and put back into the cage with their mother. Untreated control rats (n = 9), sham-operated control animals treated with DMSO implants (n = 17), DMSO + BMI-treated rats (n = 18), and DMSO + muscimol-treated rats (n = 15) were examined at P7 or P14.

**Histology and Immunohistochemistry**

After survival times of 7 or 14 days, animals were anesthetized with ethane (Abbott Laboratories, Wiesbaden, Germany) and decapitated, and the brains were removed and fixed in 4% paraformaldehyde in phosphate buffer (PB) (pH 7.4). The brains were cryoprotected in 30% sucrose in PB pH 7.4 for 24 h and serially sectioned in 50-μm coronal slices. For Nissl staining, sections were mounted on gelatin-coated slides and air-dried overnight. After incubation in cresyl violet solution for 10 min, slides were washed in water for 1 min and subsequently dehydrated in increasing concentrations of ethanol, cleared in xylene, and cover slipped. 

For immunostaining, sections were quenched with water peroxide for 30 min, blocked with 3% normal serum in PB for 1 h, and incubated with primary antibody at 4 °C overnight. After washing in phosphate-buffered saline (PBS), sections were incubated for 1 h in secondary antibody (biotinylated horse anti-mouse 1:200, biotinylated mouse anti-rabbit 1:200, Vector Laboratories, Burlingame, CA). Avidin coupled to peroxidase was used to label the secondary antibody (Vectastain ABC elite kit, Vector Laboratories), and signals were revealed by reaction of peroxidase with diaminobenzidine (DAKO, Carpinteria, CA) in 0.06% water peroxide. The following primary antibodies were used: rabbit polyclonal antibody directed against NMDA receptor 1/2ab diluted 1:2000 in 3% normal goat serum (NHS) with 0.1% Triton X-100 in PB, mouse monoclonal antibody directed against parvalbumin diluted 1:10 000 in 3% NHS (Paxinos and others 2000), rabbit polyclonal antibody directed against GABA diluted 1:5000 in 3% NHS with 0.1% Triton X-100 in PB, and monoclonal antibody directed against glial fibrillary acidic protein (GFAP) diluted 1:2000 in 3% NHS with 0.1% Triton X-100 in PB. All sections were examined under an Axioskop light microscope (Zeiss, Jena, Germany).

**5-Bromo-2'-Deoxyuridine Migration Assay in Organotypic Cortical Slices**

Organotypic neocortical slices were prepared from embryonic day (E) 18–19 Wistar rat embryos and cultivated according to the Stoppini method (Stoppini and others 1991; Haydar and others 1999). E18–19 embryos were chosen because layer II–III neurons are generated at this age (Miller and Nowakowski 1988). Pregnant animals were anesthetized by inhalation of ethane and killed by cerebral dislocation. Embryos were removed by cesarean section, and brains were isolated in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 26 NaHCO3, 3 KCl, 1.6 CaCl2, 1.8 MgCl2, 1.3 NaH2PO4, and 40 glucose, pH 7.4, at 4 °C. Coronal slices of 400-μm thickness were cut with a vibroslicer (Pelco 101, TPI, St Louis, MO, and HR2, Sigmam Elektronik, Hufnernadt, Germany), and slices containing the parietal cortex were collected in ice-cold ACSF. The meninges were carefully removed, and each hemisphere was isolated. The slices were transferred onto Millicell-CM membranes (Millipore, Bedford, MA) placed in 35-mm petri dish containing 1 ml of neurobasal medium supplemented with 2% B27 (GIBCO, Invitrogen, Carlsbad, CA). Slices were kept at 37 °C in 95% O2/5% CO2, and after 4 h, the medium was replaced by fresh medium supplemented with 10 μM 5-bromo-2'-deoxyuridine (BrdU), Sigma, St Louis, MO). Slices were cultivated in the presence of BrdU for 4 h. Then the BrdU-containing medium was replaced with fresh medium without BrdU in which 20 μM BMI (dissolved in 0.2% DMSO) or 10 μM muscimol was added. After 24 h in this medium, slices were fixed in 4% paraformaldehyde. For control experiments of BMI, an equal concentration of DMSO (0.2%) was added to control slices. These experiments demonstrated that 0.2% DMSO had no effect on neuronal migration in vitro.

For BrdU immunostaining, slices were rinsed in 10 mM PBS and treated with 0.1% Triton X-100 in PBS for 30 min followed by 2 N HCl for 90 min. Slices were incubated overnight in 3% bovine serum albumin in PBS with mouse monoclonal antibody against BrdU (1:400, Becton Dickinson, San Jose, CA), washed with PBS and then incubated for 2 h with Alexa-red-conjugated antibody against mouse (1:400, Molecular Probes, Eugene, OR). After a final wash with PBS, slices were mounted on glass slide. The slices were analyzed using a Nipkow spinning disk confocal system (Visitech, Sunderland, UK) equipped with a laser source (Laser Physics, West Jordan, UT) and an Olympus (Tokyo, Japan) microscope. Images were collected using a charge-coupled device (CCD) camera (CoolSnap HQ, Roper Scientific, Trenton, NJ). A sequence of images starting from the ventricular zone and ending at the level of the pia mater was taken with a 20× water immersion objective. Images were analyzed with Metamorph software (Universal Imaging Corporation, West Chester, PA). Migrating neurons were analyzed in the dorsolateral part of the parietal neocortex to ensure that identical neocortical regions were examined in different slices. To quantify BrdU cell distribution, the cerebral cortex was divided into 3 equal sectors from the ventricular surface to the pial surface (I–III), and the number of BrdU-positive (BrdU+) cells in each sector was manually counted using Metamorph software (Molecular Devices, Sunnyvale, CA) (Gongidi and others 2004). The percentage of BrdU+ cells present in each zone was calculated. A minimum of 10 organotypic slices from at least 3 independent experiments were analyzed per control or experimental condition. The fluorescent images were inverted and contrasted in gray-scale mode using Photoshop software (Adobe Systems, San Jose, CA) so that fluorescent signals appear in black in Figure 5.
monitored in cortical plate neurons by taking measurements every 5 s. Were obtained using a water immersion 20× objective lens (UMPlanFI, numerical aperture 0.50; Olympus) via a cooled CCD camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu, Japan) fitted to an upright microscope (BX51WI, Olympus). Data were stored for offline analysis by means of image-processing software (AquaCosmos, Hamamatsu Photonics). 

**Calcium Imaging**

The methods used for Ca\(^{2+}\) imaging were similar to those described previously (Fukuda and others 1998). Neurons in parietal cortical slices from P0 rats were loaded with the Ca\(^{2+}\) indicator Fluoro-3 by incubating slices for 60 min with Fluoro-3 acetoxyl methyl (10 μM) in ACSF containing 0.01% pluronic F127. Slices were then laid on the glass bottom of a submerged-type chamber, which was placed on a microscope stage and continuously perfused with standard ACSF gassed with 95% O\(_2\)/5% CO\(_2\) at a rate of 2–3 ml/min. The bathing solution was maintained at 30 °C and had a pH of 7.4. Fluoro-3 fluorescence was excited (bandpass 460–480 nm) using a mercury lamp fitted to a multislit disk confocal scanner (BX-DSU; Olympus), and the emitted light was filtered using a band-pass filter (BA 495–540 nm). Confocal fluorescence images were obtained using a water immersion 20× objective lens (UMPlanFI, numerical aperture 0.50; Olympus) via a cooled CCD camera (ORCA-ER-1394, Hamamatsu Photonics, Hamamatsu, Japan) fitted to an upright microscope (BX51WI, Olympus). Data were stored for offline analysis by means of image-processing software (AquaCosmos, Hamamatsu Photonics). 

\[\text{[Ca}^{2+}\text{]}_\text{im}\]

Signals were expressed as the ratio of the Fluoro-3 fluorescence intensity changes compared with baseline (E/FO). Signals were monitored in cortical plate neurons by taking measurements every 5 s. All data are presented as mean ± standard error of the mean. For statistical analysis the Student’s *t*-test or the unpaired *t*-test and analysis of variance (ANOVA) were used.

**Results**

**Drug Release from Elvax Slices**

In order to analyze the kinetics of BMI and muscimol release from the Elvax implants, we measured over a period of 16 days by scintillation counting the release of radioactive-labeled BMI and muscimol (Fig. 1). These Elvax slices were identical in size (1 mm\(^2\), 100-μm thickness) as those used in the in vivo experiments. In good agreement with previous observations (Schnupp and others 1995; Smith and others 1995; Reiprich and others 2005), BMI as well as muscimol was released predominantly during the first 2 days. Because previous reports have documented that the drug release characteristics of slices implanted in the brain are very similar to those incubated at 37 °C in vitro (Smith and others 1995), these data indicate that the release of BMI and muscimol from slices embedded in newborn rat cortex is mostly restricted to the first 2 postnatal days.

**GABA-A Receptor Modulation Induces Neuronal Migration Disorders In Vivo**

After survival times of 7 or 14 days, the cerebral cortex of control and experimental animals was Nissl stained; immunoreacted for NMDA, GABA, parvalbumin, or GFAP; and analyzed in detail by the use of a light microscope. All cortices from untreated controls (*n* = 9) and sham-operated animals treated with DMSO implants (*n* = 17) showed the normal cortical architecture (Fig. 2). By comparing the untreated animals with those of the DMSO group, we assured that the solvent had no influence on cortical development. The cortices of both control groups showed the typical 6-layered organization, and abnormalities were never seen in these animals.

In contrast to the control animals, the cortex of all BMI- (*n* = 18) and muscimol-treated (*n* = 15) rats revealed prominent structural abnormalities, which were restricted to the site of the Elvax implants, indicating that these cortical malformations were induced by the release of the drugs during the first postnatal days. These structural abnormalities were characterized by severe disturbances in cortical layering, focal cortical dysplasias, and heterotopia within the upper cortical layers often extruding from the pial surface (Fig. 3). The cortex of BMI-treated animals (Fig. 3A) as well as muscimol-treated rats (Fig. 3D) showed ectopic cell clusters in layer I, suggesting that neurons migrated directly to the pial surface instead of stopping at their proper positions.

In order to characterize in more detail the cellular composition of the upper layer heterotopia observed with Nissl staining in BMI- and muscimol-treated animals, we performed immunohistochemical stainings on adjacent sections. Immunostaining...
for the NMDA receptor allowed the identification of non-pyramidal and pyramidal neurons in the heterotopia (Fig. 4A). As shown in the inset of Figure 4A2, some pyramidal cells in the heterotopia showed an atypical somatodendritic orientation. The distribution of interneurons in the heterotopia was assessed by the use of an antibody directed against GABA (Fig. 4B). GABA-labeled interneurons were present within the upper layers heterotopia, and a similar distribution could be confirmed with the marker parvalbumin (data not shown). Hence, both glutamatergic and GABAergic neurons were present within the affected cerebral cortex. In order to rule out the possibility that neocortical heterotopia could be due to glial cell proliferation, we also performed immunostaining for the astrocytic marker GFAP. These sections showed the typical GFAP localization in the vicinity of the pia mater, where the glia limitans is formed, but no GFAP-positive, activated or proliferative astrocytes within the heterotopia (Fig. 4C).

To our surprise, the GABA-A receptor antagonist BMI and the GABA-A receptor agonist muscimol both induced very similar cortical malformations in vivo. Whereas previous in vitro studies have already demonstrated that blockade of GABA-A receptors induces migration disturbances in neocortical neurons (Behar and others 2000), our finding that the GABA-A receptor agonist muscimol also induces deficits in neuronal migration is new. In order to understand in more detail the effects of BMI and muscimol application on the neuronal migration process in the perinatal rat parietal cortex, we used an in vitro assay.

**Neuronal Migration In Vitro**

The functional role of the GABA-A receptor in neuronal migration was studied in vitro using organotypic neocortical slice cultures from E18–19 rat embryos and a BrdU-based migration assay (Haydar and others 1999). In all control slice cultures (n = 22), BrdU pulse labeling of migrating neurons destined for the upper cortical layers revealed the typical migration pattern as expected from previous studies (Behar and others 2000). After 24 h in culture, an ~250-µm-wide band of BrdU+ cells was located in sector II and the lower part of sector III (Fig. 5A1) with a significantly lower number of cells in sector III as compared with sector II. In good agreement with previous observations by Behar and others (2000), blockade of GABA-A receptors with 20 µM BMI for 24 h caused a significant (P < 0.05) enhanced migration of BrdU+ cells into the cortical plate (Fig. 5A2, B1). In BMI-treated slices (n = 10), the band of migrating BrdU+ neurons was largely located in sector III and the upper part of sector II, whereas in controls, the majority of migrating cells were found in sector II. A similar picture emerged from slice cultures treated for 24 h with the GABA-A agonist muscimol (10 µM, n = 14) (Fig. 5A3). Although the quantitative analysis between controls and muscimol-treated slices did not reveal a statistically significant difference at the P < 0.05 level, a significantly lower number of BrdU+ cells were counted in sector III compared with sector II in control slices (P < 0.01, ANOVA), whereas BrdU+ cells were equally distributed in sector II and III in muscimol-treated slices (P > 0.05, ANOVA), indicating that muscimol also tended to promote the migration rate.

In order to understand the acute effects of BMI and muscimol application on migrating neurons and because neuronal migration is strongly regulated by the [Ca2+]i concentration (Komuro and Rakic 1992; Soria and Valdeolmillos 2002; Kumada and Komuro 2004), we investigated the effects of BMI and muscimol...
application on spontaneous \([\text{Ca}^{2+}]_i\) oscillations in neocortical slices from newborn rats.

**Effects of Acute Bicuculline and Muscimol Application on Spontaneous Calcium Oscillations**

In agreement with previous studies (Yuste and Katz 1991), neocortical slices from P0 animals revealed spontaneous \([\text{Ca}^{2+}]_i\) oscillations at a frequency of 26.2 ± 1.0 per h (\(n = 374\) cells, \(n = 19\) slices), which were synchronized in a large number of neurons (Garaschuk and others 2000). These spontaneous \([\text{Ca}^{2+}]_i\) oscillations were completely blocked or significantly reduced in frequency by bath application of BMI at a concentration of 20 \(\mu M\) (from 36 ± 5.4 to 17.6 ± 3.7 per h, \(n = 42\) cells, 5 slices, \(P < 0.01\), Fig. 6A1) and 5 \(\mu M\) (from 22.5 ± 0.9 to 4.8 ± 0.7 per h, \(n = 91\), 2 slices, \(P < 0.001\), Fig. 6A2) without affecting the resting \([\text{Ca}^{2+}]_i\). These data suggest that spontaneous \([\text{Ca}^{2+}]_i\) oscillations in the cerebral cortex of newborn rats depend on GABA-A receptor activation. Prolonged application of muscimol (10 \(\mu M\)) induced an initial increase in \([\text{Ca}^{2+}]_i\) that returned to baseline level within 3–5 min (4 slices) (Fukuda and others 1998). This initial \([\text{Ca}^{2+}]_i\) response to muscimol was followed by a significant reduction in the frequency of spontaneous \([\text{Ca}^{2+}]_i\) oscillations from 41.7 ± 5.2 to 8.2 ± 2.2 per h (\(n = 31\) cells, 4 slices, \(P < 0.001\), Fig. 6B1). A similar response could be obtained with a lower muscimol concentration of 1 \(\mu M\), which also induced a significant decrease in the spontaneous \([\text{Ca}^{2+}]_i\) oscillations from 17.5 ± 0.8 to 4.9 ± 0.6 per h (\(n = 89\) cells, 4 slices, \(P < 0.001\), Fig. 6B2). At these
concentrations, BMI and muscimol had different effects on the initial baseline $[\text{Ca}^{2+}]_i$, but both abolished the spontaneous $[\text{Ca}^{2+}]_i$ oscillations.

In order to understand the mechanisms that may underlie this muscimol effect, we investigated with electrophysiological methods in neocortical slices from newborn rats the responses of migrating neurons to prolonged application of muscimol.

**Functional Interaction between Muscimol and GABA-A Receptors in Migrating Neurons**

We performed whole-cell patch-clamp recordings from migrating neurons in the cortical plate of P0–1 rats. The recorded neurons displayed the typical morphological features of migrating neurons (Fig. 7A,B) and had an average resting membrane potential of $-61.1 \pm 2.5$ mV ($n=24$) and an input resistance of $2.6 \pm 0.3$ GOhm. In 15 of the 24 analyzed neurons, action potentials with a small amplitude ($28.9 \pm 2.1$ mV) and long duration ($9.5 \pm 0.9$ ms) could be recorded. These data are in good agreement with previous electrophysiological studies on migrating neurons in newborn rat cerebral cortex (Luhmann and others 2000). Short pulses of GABA (1 mM, 3–5 ms) were pressure applied focally and evoked fast inward current responses with an average amplitude of $-157.2 \pm 20.5$ pA ($n=15$) (downward deflections and lower row in Fig. 7C,D). In addition, the GABA-A receptor agonist muscimol was bath applied to investigate its effect on GABA-induced currents. The application of 10 μM muscimol induced an inward current of $-80.5 \pm 10.6$ pA ($n=6$), which desensitized by $61.3 \pm 5.1\%$ (Fig. 7C). The inward currents induced by the focal GABA application were almost completely abolished by $97.2 \pm 0.9\%$ in the presence of 10 μM muscimol. Application of 1 μM muscimol induced an inward current of $-33.3 \pm 11.2$ pA ($n=9$), which desensitized by $39.5 \pm 7.1\%$ (Fig. 7D). Under this condition, the GABA-induced inward currents were reduced by $82.4 \pm 3.1\%$ ($n=9$). These data demonstrate that prolonged application of muscimol, as during the 2-day release period of muscimol from the Elvax slices in vivo, significantly reduces the activation of GABA-A receptors due to pronounced receptor desensitization.

**Discussion**

Our in vivo and in vitro findings demonstrate that the neuronal migration process of neocortical neurons is strongly regulated by GABAergic mechanisms. Previous in vitro studies have already shown that GABA-A as well as GABA-B receptors modulate the migration process of cortical neurons (Behar and others 1996, 2000, 2001; López-Bendito and others 2003). Using an in vitro migration assay, Behar and others (1996) demonstrated that the GABAergic modulation of neuronal migration is strongly concentration dependent. Whereas femtomolar concentrations of GABA stimulated the migration along a chemical gradient (chemotaxis), micromolar GABA caused an increased random migratory cell movement (chemokinesis). Both processes were mediated by an $[\text{Ca}^{2+}]_i$ increase, indicating that GABA may mediate both chemotactic and chemokinetic migratory responses in embryonic neocortical neurons (for review, Barker and others 1998). Interestingly, we observed distinct pathological alterations in the cortical architecture of animals treated with the GABA-A antagonist BMI or the agonist muscimol in vivo. Pyramidal neurons and GABAergic interneurons were present in high density in heterotopic cell clusters in the upper cortical layers. This heterotopia often protruded out of the cortical surface and could be easily detected in the Nissl stains. Because such cortical malformations were observed neither in the control animals nor in the untreated hemisphere of the BMI- or muscimol-treated rats and because these...
Malformations were restricted to the site of the Elvax implant; these neuronal migration disorders most likely result from the drug-induced interference with the GABA-A receptor. Our in vivo data indicate that BMI as well as muscimol causes an enhancement in the neuronal migration process that leads to the clustering of neurons in the normally cell-sparse layer I and to the focal cortical protrusion. A migration-promoting effect of bicuculline has been previously demonstrated in vitro by Behar and others (2000), indicating that GABA-A receptor activation may act as a "stop" signal for migrating neocortical neurons. Both, our in vivo and our in vivo data are in good agreement with this previous report on organotypic cortical slice cultures, and we further demonstrate that this bicuculline effect on neocortical neuronal migration most likely results from the

Figure 5. Role of GABA-A receptors in neocortical neuronal migration in vitro. Organotypic parietal cortical slices incubated with BrdU for 4 h and maintained in culture for 24 h show typical migration pattern of BrdU+ cells destined for upper cortical layers (A1). Blockade of GABA-A receptors with 20 μM BMI causes a significant increase in the migration rate (A2) compared with the untreated controls, as also documented in the quantitative analysis of migrating neurons in the 3 zones I–III (B1). Application of 10 μM muscimol induces a smaller and nonsignificant increase in the migration rate (A3, B2). Data in (B) are expressed as mean ± standard error of the mean and are based on counts of ~1000 BrdU+ cells per slice and on 10-14 slices from 3 independent cultures per control or experimental group. Scale bar in (A2) corresponds to 100 μm.
Figure 6. Effects of the GABA-A receptor antagonist BMI (A) and the agonist muscimol (B) on spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in the cortical plate of P0 rats. Each trace shows typical responses of spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} oscillations to either compound at 2 different concentrations. Bath applications of BMI (20 and 5 μM) or muscimol (10 and 1 μM) completely blocked or significantly reduced the spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. Note that both compounds differ in their initial effects on resting [Ca\textsuperscript{2+}]\textsubscript{i}, but both cause a long-lasting suppression of spontaneous Ca\textsuperscript{2+} transients.

blockade of spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} transients. The neuronal migration process in the cerebellum (Komuro and Rakic 1992, 1998) as well as in the cerebral cortex (Behar and others 1996; Barker and others 1998) is strongly regulated by the [Ca\textsuperscript{2+}]\textsubscript{i} concentration, and it has been recently demonstrated that the [Ca\textsuperscript{2+}]\textsubscript{i} transients control the migration process (Kumada and Komuro 2004; Komuro and Kumada 2005). We show that these [Ca\textsuperscript{2+}]\textsubscript{i} transients are not only blocked by bicuculline but also by muscimol at 2 different concentrations. Our in vitro electrophysiological studies demonstrate that this muscimol effect can be attributed to a prominent GABA-A receptor desensitization. Prolonged application of the GABA-A agonist muscimol causes a suppression of postsynaptic GABAergic responses. Our results are in good agreement with recent in vitro observations demonstrating in acute mouse brain slices that application of a desensitizing concentration of GABA significantly increased the speed of cell migration by 39% (Bolteus and Bordey 2004). Application of bicuculline enhanced the migration speed by 35%, suggesting that endogenous GABA tonically reduces the speed of cell migration via GABA-A receptor activation and by interfering with intracellular Ca\textsuperscript{2+} signaling (Bolteus and Bordey 2004). Our data indicate that in vivo application of a GABA-A receptor antagonist or a GABA-A agonist of sufficiently high concentration to induce receptor desensitization may also increase migration speed in the neurons located under the Elvax implant. This enhancement in migration speed may lead to the heterotopia that we observed in the upper cortical layers of BMI- and muscimol-treated animals. Our in vitro observations are compatible with this hypothesis.

Beside interfering with the tonic, paracrine action of GABA during early development (Demarque and others 2002), application of BMI (Schwartz and others 1998; Opitz and others 2002) or muscimol (present study) also blocks spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} transients in immature cerebral cortex. Because intracellular Ca\textsuperscript{2+} represents a key element in the regulation of the neuronal migration process (Komuro and Rakic 1992; Soria and Valdeolmillos 2002; Kumada and Komuro 2004), the cortical malformations demonstrated in our experiments may have been also caused by a GABA-A-mediated disturbance of spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. It is well established that activation of GABA-A receptors has a depolarizing effect in immature cortical neurons (Ben-Ari and others 1989; Luhmann and Prince 1991; for review, Ben-Ari 2002). In rodents, this depolarizing action of GABA changes at the end of the 1st postnatal week into a hyperpolarizing action due to a developmental upregulation of chloride-extruding mechanisms via the K\textsuperscript{+}/Cl cotransporter KCC2 (Rivera and others 1999) and a simultaneous downregulation of the chloride-outward Na-K-Cl cotransporter NKCC1 (Yamada and others 2004). Although the exact downstream mechanisms on the regulation of GABA-mediated [Ca\textsuperscript{2+}]\textsubscript{i} rises on neuronal migration speed are currently not known, our data and previous observations (Bolteus and Bordey 2004) indicate that GABA-A receptor activation profoundly regulates the migration process by altering [Ca\textsuperscript{2+}]\textsubscript{i} levels.

We cannot exclude that our in vivo approach also interferes with other important processes during early cortical development. Spontaneous neuronal activity and synchronized [Ca\textsuperscript{2+}]\textsubscript{i} transients in Cajal-Retzius cells and other layer I neurons (Schwartz and others 1998; Aguilo and others 1999) may be also modified by the application of GABA-A agonists or antagonists. Cajal-Retzius neurons play a pivotal role in controlling neuronal migration because they release the extracellular matrix protein reelin (for review, Marin and Rubenstein 2003). Spontaneous synaptic activity onto Cajal-Retzius neurons is mediated by GABA-A receptors (Kilb and Luhmann 2001; Soda and others 2003), and modulation of this receptor may interfere with the release of reelin or the migration process. A functional silencing of Cajal-Retzius cells might have similar consequences as an early ablation of these neurons (Super and others 2000).

We conclude that modulation of GABA-A receptor-mediated mechanisms in the immature cerebral cortex impairs neuronal migration in not only various in vitro models but also in vivo models. The resulting pathological alterations in the
cortical architecture resemble neuronal migrations disorders described in human cerebral cortex (for review, Gleeson and Walsh 2000). Our data also imply that drugs acting during early developmental stages on GABA-A receptors, GABA metabolic pathways (Pearl and others 2004), or GABA transporters (Conti and others 2004) may have profound side effects on the structural and functional development of the cerebral cortex.

Figure 7. Effect of prolonged muscimol application on GABA-A responses in migrating neurons of the newborn rat. (A) Photomicrograph of a biocytin-filled migrating neuron in the cortical plate of a P1 rat. (B) Same cell as in (A) at higher magnification. (C, D) Whole-cell voltage-clamp recordings from migrating neurons (P0-1) illustrating responses to focal pulse application of GABA (short downward current deflections) and to bath application of 10 μM (C) or 1 μM (D) muscimol. Note that GABA-induced inward currents are strongly reduced in the presence of muscimol. The current responses marked by asterisks are displayed at higher temporal resolution in the bottom traces.
**Notes**

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