GABA$_C$ RECEPTORS ARE FUNCTIONALLY EXPRESSED IN THE INTERMEDIATE ZONE AND REGULATE RADIAL MIGRATION IN THE EMBRYONIC MOUSE NEOCORTEX

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Abstract—Radial neuronal migration in the cerebral cortex depends on trophic factors and the activation of different voltage- and ligand-gated channels. To examine the functional role of GABA$_C$ receptors in radial migration we analyzed the effects of specific GABA$_A$ and GABA$_C$ receptor antagonists on the migration of BrdU-labeled neurons in vitro using organotypic neocortical slice cultures. These experiments revealed that the GABA$_A$ specific inhibitor bicuculline methiodide facilitated neuronal migration, while the GABA$_C$ specific inhibitor (1,2,5,6-tetrahydropyridine-4-yl) methylphosphinic-acid (TPMPA) impeded migration. Co-application of TPMPA and bicuculline methiodide or the unspecific ionotropic GABA receptor antagonist picrotoxin revealed that the GABA$_C$ receptor mediated effects dominate. Addition of the specific GABA$_C$ receptor agonist cis-4-aminocrotonic acid (CACA) also hampered migration, indicating that a physiological GABAergic stimulation is required for appropriate function. RT-PCR experiments using specific probes for GABA$_A$ receptor mRNA and Western blot assays using an antibody directed against rho subunits revealed the expression of GABA$_A$ receptor mRNA and translated GABA$_A$ receptor protein in the immature cortex. Microfluorimetric Ca$^{2+}$ imaging in neurons of identified cortical layers using Calcium Green revealed the functional expression of GABA$_A$ and GABA$_C$ receptors in the intermediate zone, while only GABA$_A$ receptor mediated responses were observed in the upper cortical plate. In summary, these results demonstrate that activation of GABA$_C$ receptors is a prerequisite for accurate migration and that GABA$_C$ receptors are functionally expressed in the intermediate zone. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: neuronal migration, organotypic slice culture, GABA$_C$ receptor, GABA pharmacology, calcium imaging, cortical development.

The development of the CNS involves a complex sequence of events (Goodman and Shatz, 1993; Bystron et al., 2008), which are regulated by genetic factors, intrinsic activity patterns and sensory information (Edlund and Jessell, 1999; Sur and Rubenstien, 2005; Nobrega-Pereira and Marin, 2009). The migration of neurons from their birthplace to their final destination is an important step in brain development and can be classified into radial, tangential and chain migration (Manent and Represa, 2007). For radial migration in mammalian neocortex, pyramidal neurons are generated in the ventricular and subventricular zone and migrate radially through the intermediate zone to reach the cortical plate, where they differentiate into their final phenotype and incorporate into the neuronal network (Kriegstein and Noctor, 2004; Rakic, 2006). An increasing body of evidence suggests that neuronal activity participates in the regulation of several aspects of early neuronal development like proliferation, migration or neuronal differentiation even before the establishment of neuronal networks (LoTurco et al., 1995; Komuro and Rakic, 1998; Spitzer, 2006; Elias and Kriegstein, 2008; Root et al., 2008). Pioneer studies performed on cerebellar granule cells showed that neural activity through activation of ligand-gated and voltage-dependent ion channels could also be involved in the regulation of neuronal migration (Komuro and Rakic, 1992). This observation has been widened and it is now clear that a variety of neurotransmitters are involved in the regulation of migration of olfactory bulb neurons (Bolteus and Bordey, 2004b), hippocampal neurons (Manent et al., 2005), GABAergic interneurons (Lopez-Bendito et al., 2003; Cuzon et al., 2006) and cortical principal neurons (Behar et al., 2000; Reiprich et al., 2005; Heck et al., 2007; Ayala et al., 2007; Manent and Represa, 2007).

Previous in vitro and in vivo studies have demonstrated that GABA is particularly implicated in the regulation of neuronal migration, acting as chemotactic agent, as regulator of cell mobility and mediator of the migration process (Behar et al., 1994, 1996, 1998, 2000; Heck et al., 2007). Ionotropic GABA receptors are already expressed in cortical neuroblasts at proliferative stages in the ventricular zone (LoTurco et al., 1995). Since migrating neurons do not form chemical synapses, it is considered that extracellular GABA activates the postsynaptic receptors in a paracrine manner (Demarque et al., 2002; Manent et al., 2005). During early developmental stages neocortical neurons maintain a high intracellular Cl$^-$ concentration leading to a Cl$^-$ efflux and membrane depolarization upon activation of ionotropic GABA receptors (Luhmann and Prince, 1991; Owens et al., 1996; Yamada et al., 2004; Achilles et al., 2007; Ben-Ari et al., 2007). This depolarization conse-
quenty leads to opening of voltage-dependent Ca\(^{2+}\) channels and Ca\(^{2+}\) influx (Owens et al., 1996). Inhibition of GABA\(_A\) receptors in vivo induces severe malformations of the cortical layers leading to upper cortical heterotopia (Heck et al., 2007). Migration assays in organotypic slice cultures showed that inhibition of GABA\(_A\) receptors improves migration (Behar et al., 2000; Heck et al., 2007), which could explain this in vivo observation. In contrast, the GABA\(_A\)/GABA\(_C\) antagonist picrotoxin impedes radial migration (Behar et al., 2000). On the basis of these controversial effects, a distinct role for ionotropic GABA\(_C\) receptors in regulating neuronal migration was proposed. The specific effects of different GABAergic antagonists were integrated in a model in which ionotropic and metabotropic GABA receptors regulate successive steps of neuronal migration (Behar et al., 2000; for review see Owens and Kriegstein, 2002; Manent and Represa, 2007; Heng et al., 2007).

However, to our knowledge the role of GABA\(_A\) receptors in radial migration was experimentally not directly addressed. Moreover, there is so far no direct evidence for the functional expression of GABA\(_A\) receptors in the embryonic neocortex. Therefore we investigated the questions (i) how GABA\(_A\) receptors affect radial migration and (ii) whether they are functionally expressed in the developing mouse cerebral cortex. We provide evidence for a direct effect of GABA\(_A\) receptors on radial migration and for a functional expression of GABA\(_A\) receptors in the intermediate zone, which further supports the model for a regulatory role of different GABA receptor subtypes in neuronal migration.

**EXPERIMENTAL PROCEDURES**

**Migration assay**

All experiments were conducted in accordance with the national laws for the use of animals, the EC guideline for the ethical use of animals in research (86/609/EEC) and approved by the local ethical committee (Landesuntersuchungsanstalt RLP, Koblenz, Germany, #23.05-230-3-76/00). All efforts were made to minimize the number of animals used and their suffering. Organotypic neocortical slices were prepared from embryonic day (E) E17–18 C57Bl/6 mouse embryos and cultivated according to the Stoppini method (Stoppini et al., 1991; Haydar et al., 1999). Pregnant animals were anesthetized by inhalation of ethrane and killed by cervical dislocation. Embryos were removed by cesarean section (E17–18 C57Bl/6 mouse embryos and were used acutely or after 1 day in vitro. Neurons were loaded with the Ca\(^{2+}\) indicator Calcium Green-1, AM (Molecular probes) by incubating slices for 15 min with Calcium Green-1 (1.25 \(\mu\)M) in the presence of 0.01% pluronic F127 (Molecular Probes) in ACSF (composition see below). Slices were placed on the glass bottom of a submerged-type chamber, placed under a microscope (Olympus BX51WI) equipped with a confocal Nipkow spinning disk confocal system (Visitech, Sunderland, UK) equipped with a laser source (Laser Physics, West Jordan, UT, USA) on an Olympus (Tokyo, Japan) microscope using appropriate filter sets for the 488/568 nm lines of the Kri/Ar laser (Visitech). Images were collected by a CCD camera (CoolSnap HQ; Roper Scientific, Trenton, NY, USA) using Metamorph software. Migrating neurons were visualized in the dorso-lateral 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 three equal sectors from the ventricular surface to the pial surface (I to III) and the number of BrdU-positive cells in each sector was counted (Gongidi et al., 2004; Heck et al., 2007). The percentage of BrdU-positive cells in each sector (divided by the total number of BrdU-positive cells) was calculated. Since the cortical orientation is severely impaired in the experiments with distorted neuronal migration, these sectors could not directly be linked to the cortical layers. In control slices after 2 days in vitro (DIV) section I approximately covers the ventricular/subventricular zone and part of the intermediate zone, section II corresponds to the remaining intermediate zone, the subplate and the lower cortical plate, while sector III covers the upper cortical plate and the marginal zone (Suppl. Fig. 1). All data are presented as mean±standard error of the mean (SEM). For statistical analysis the Mann–Whitney test (Prism, GraphPad, LaJolla, CA, USA) was used. Significance was assigned at levels of 0.05 (*), 0.01 (**) and 0.001 (***)

**Calcium imaging**

Neocortical slices of 200–300 \(\mu\)m thickness were prepared from E17–18 C57Bl/6 mouse embryos and were used acutely or after 1 day in vitro. Neurons were loaded with the Ca\(^{2+}\) indicator Calcium Green-1, AM (Molecular probes) by incubating slices for 15 min with Calcium Green-1 (1.25 \(\mu\)M) in the presence of 0.01% pluronic F127 (Molecular Probes) in ACSF (composition see below). Slices were placed on the glass bottom of a submerged-type chamber, placed under a microscope (Olympus BX51WI) equipped with a confocal Nipkow spinning disk confocal system (see above) and were continuously superfused with ACSF maintained at 32 °C at a rate of 3 ml/min. Experiments started after wash of excess Calcium Green-1 for 20 min. Slices were viewed through an immersion objective (20\(\times\), NA 0.5, Olympus) and images were acquired every 3 s for a 4 min interval using a CCD camera controlled by the Metamorph software. GABA or cis-4-aminoacrotonic acid (CACA) were applied for 1 min after a 15 s baseline interval and were subsequently washed out for 30 min. Antagonist perfusion started 10 min before data acquisition, the
reversibility of GABA effects was determined at least 25 min after washout of the antagonists. Ca$^{2+}$ transients induced by 4 mM glutamate or 50 mM K$^+$ were used to test for viability after blockade of GABA_A and GABA_C receptors. Ca$^{2+}$ responses were expressed as the ratio of the Calcium Green-1 fluorescence intensity changes compared to baseline (F/F_0). Only fluorescence changes at least four times larger than the standard deviation of the baseline interval (15 s) were considered as Ca$^{2+}$ responses. All data are presented as mean±SEM. For statistical analysis Wilcoxon signed-ranks test and Fisher’s exact test (two-tailed) were used (Systat 11, Point Richmond, CA, USA). Significance was assigned at levels of 0.05 (*), 0.01 (**) and 0.001 (***)

**Solutions and drugs**

For acute slice experiments ACSF consisted of (in mM) 126 NaCl, 26 NaHCO_3, 1.25 NaH_2PO_4, 1 MgCl_2, 2 CaCl_2, 2.5 KCl, 10 glucose (pH 7.4, osmolarity 306 mOsm) equilibrated with 95% O_2/5% CO_2. For cultured slices ACSF consisted of (in mM) 51 NaCl, 26 NaHCO_3, 0.9 NaH_2PO_4, 0.8 MgCl_2, 1.8 CaCl_2, 5.3 KCl, 25 glucose and 11 HEPES (pH 7.4, osmolarity 306 mOsm) equilibrated with 95% O_2/5% CO_2. For pharmacological experiments γ-aminobutyric acid (GABA, Sigma), picrotoxin (Sigma), (-)-bicuculline methiodide (BMI, Sigma), (1,2,5,6-tetrahydropyridine-4-yl)methylphosphonic-acid (TPMPA, Tocris Cookson, Bristol, UK) and CACA (Sigma), were used from stock solutions in distilled water (GABA, TPMPA, CACA) or DMSO (BMI). All stock solutions were stored at −20 °C and diluted to the final concentration on the day of experiment. DMSO concentration never exceeded 0.1%. Since the effects of antagonists were not significantly different between acute and cultured slices, data were pooled.

**RNA isolation and reverse transcription polymerase chain reaction**

For reverse transcription polymerase chain reaction (RT-PCR) experiments, total RNA was purified from E18 mouse cortex, frozen in liquid nitrogen and purified using RNeasy Micro Kit (Qiagen, Hilden, Germany). Reverse transcription was performed with SuperScriptIII Reverse Transcriptase Kit (Invitrogen). 50 ng of total RNA and 0.2 mM deoxyribonucleoside triphosphates (dNTPs) and 50 μM OligodT were mixed in a total volume of 29 μl, incubated at 65 °C for 5 min and cooled down 1 min on ice. Afterwards 4 μl 5× First-Strand Buffer, 1 μl 0.1 M dithiothreitol, 1 μl RNaseOut (40 units/μl) and 1 μl of SuperScriptIII RT (200 units/μl) were added. The reactions were incubated for 60 min at 55 °C, followed by an inactivation step for 15 min at 70 °C. Amplification of the cDNA was carried out using the QIAGEN Taq PCR Kit. 10 μl of the appropriate 10 mM sense and antisense primer (Invitrogen) were mixed cDNA of first-strand reaction. The volumes of cDNA used for detection of beta and rho subunits were 2 and 16 μl, respectively. The PCR mixture contained 10 μl 10× PCR Buffer, 4 μl MgCl_2, 20 μl 5× Q-Solution, 2 μl dNTPs (10 mM each), 0.5 μl Taq-Polymerase (2.5 units/reaction) and the cDNA-primer solution, in a total volume of 100 μl. RT-PCR experiments were performed using a thermocycler (Mastercycler Personal, Eppendorf) starting with an initial denaturation of 3 min at 94 °C, followed by cycles of 45 s at 94 °C, annealing steps of 30 s at 55 °C, extension steps of 1 min at 72 °C and a final extension step at 72 °C for 10 min. The following primer pairs were used to amplify the fragments:

- **GABA_A beta1 subunit:** forward GACAGACCTGCTCCTCGGAAATCG; reverse GGGTTTCTCTCGGTGTCAACC;
- **GABA_A rho1 subunit:** forward GGTTGTCTCCTCGGTGTCAACC; reverse GAGATGTGGTTGTTGAGCC;
- **GABA_A rho2 subunit:** forward ACAGACCTGCTCCTCGGAAATCG; reverse GGGTTTCTCTCGGTGTCAACC;
- **GABA_A beta1 subunit:** reverse TGTCCTCCTCTCGGTGTCAACC;
- **GABA_A rho2 subunit:** reverse AGAGATGTGGTTGTTGAGCC;
- **GABA_C beta1 subunit:** forward ACAGCTCCAATGAACTCAGCA; reverse TGTCCTCCTCTCGGTGTCAACC;
- **GABA_C beta1 subunit:** reverse TGTCCTCCTCTCGGTGTCAACC;
- **GABA_C rho2 subunit:** reverse AGAGATGTGGTTGTTGAGCC;
- **GABA_C rho1 subunit:** forward GACAGACCTGCTCCTCGGAAATCG; reverse GGGTTTCTCTCGGTGTCAACC;

**Western blot analysis**

Murine embryonal cortices (E17) and adult brains were homogenized with a QiAshredder (Qiagen) in chilled lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X100) supplemented with Complete® Protease Inhibitor Cocktail (Roche Applied Science). Protein concentrations were determined using the Pierce BCA™ assay (Thermo Scientific, Bonn, Germany). Proteins were separated on a 10% SDS polyacrylamide gel and transferred to a polyvinylidenefluoride membrane (Roth, Karlsruhe, Germany). The membrane was blocked for 30 min in 4% nonfat dry milk in Tris-buffered saline (TBS) with 0.1% (v/v) Tween 20 (TBST). After incubation with the rabbit primary rho63 polycional antibody against μ1, μ2 and μ3 subunits kindly provided by Prof. H. Wäsle (Max-Planck-Institut für Hirnforschung, Frankfurt a.M., Germany, see Enz et al., 1996) overnight at 4 °C (1:500 dilution in blocking solution), the membrane was washed three times with TBST and incubated with horseradish peroxidase conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA, USA) (1:5000 dilution in blocking solution) for 35 min at room temperature. After incubation, the membranes were washed three times with TBST and once in TBS. Proteins were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Schwabach). Protein sizes were compared with Precision Plus Protein Marker (Dual Colour Stand, Biorad, Munich, Germany) and Wide Range Marker (Sigma). Images were acquired using a LAS3300 imaging system (Fujifilm, Duesseldorf, Germany).

**RESULTS**

GABA_A and GABA_C receptors have opposite effects on radial migration

To investigate the effect of selective inhibition of GABA_A and GABA_C receptors on radial migration we used a BrdU-based in vitro migration assay of organotypic neocortical slice cultures from E17–18 mouse embryos. Application of 100 μM picrotoxin, a blocker of both GABA_A and GABA_C ionotropic receptors, significantly hampered neuronal migration (n=25 slices, Fig. 1A). The relative number of BrdU-labeled neurons in the upper two sections of the cortex was significantly (P<0.0001 in section II, P=0.0287 in section III) reduced as compared to controls (n=49 slices), while significantly (P<0.0001) more BrdU-labeled neurons remained in the lowest section I (Fig. 1A). In order to differentiate between the role of GABA_A and GABA_C receptors in radial migration, selective antagonists for these receptors were used. Blockade of GABA_A receptors with 100 μM BMI, an antagonist of GABA_A, but not GABA_C receptors (Bormann, 2000; Behar et al., 2000), slightly enhanced radial migration (Fig. 1A). In the continuous presence of BMI, significantly (P=0.0044) more BrdU-labeled neurons were observed in the upper section III of the cortex as compared to untreated control cortices.
Fig. 1. GABA<sub>A</sub> and GABA<sub>C</sub> receptors have opposite effects on neuronal radial migration in mouse neocortical slice cultures. (A<sub>1</sub>) Representative examples of the distribution of BrdU-labeled cells in the dorsolateral part of the somatosensory cortical slice cultures under control conditions (left), in the presence of 100 μM picrotoxin (middle) and 100 μM bicuculline methiodide (BMI, right). The cerebral cortex was divided into three sections, indicated by the dashed lines and roman numbers. (A<sub>2</sub>) Picrotoxin significantly reduces the number of BrdU-labeled cells in the upper two sections II and III, while the number of cells in the lower section I is increased. (A<sub>3</sub>) BMI induces the opposite effects, significantly increasing the number of BrdU-labeled cells in section III. (B<sub>1</sub>) Representative examples of the distribution of BrdU-labeled cells under control conditions (left), in the presence of 50 μM TPMPA (middle) or 50 μM TPMPA plus 100 μM BMI (right). (B<sub>2</sub>) In the presence of 50 μM TPMPA the number of BrdU-labeled cells in the upper two sections II and III is significantly reduced, while the number of cells in the lower section I is increased. (B<sub>3</sub>) 50 μM TPMPA plus 100 μM BMI also significantly reduce migration. The number of BrdU-labeled cells in region I is increased, while less BrdU-labeled cells are found in region III. (C<sub>1</sub>) Representative examples of the distribution of BrdU-labeled cells under control conditions (left) and in the presence of 100 μM CACA (right). (C<sub>2</sub>) CACA reduces the number of migrating neurons in the upper two sections II and III, while the number of cells in the lower section I is increased. Scale bar represents 100 μm. Columns represent mean±SEM. Mann–Whitney test, * P<0.05; ** P<0.01; *** P<0.001, ns=P>0.05.
Expression of GABA<sub>C</sub> receptors in the immature neocortex

We studied the expression pattern of GABA<sub>C</sub> receptor mRNA in E18 mouse somatosensory cortices using RT-PCR. GABA<sub>C</sub> receptors are composed of rho subunits, of which rho1 and rho2 have been described and cloned in the mouse (Greka et al., 1998; Enz, 2001). The RT-PCR analysis revealed the expression of rho1 and rho2 mRNA (Fig. 2A), demonstrating that GABA<sub>C</sub> receptor subunits are indeed expressed in the embryonic mouse neocortex. To examine whether the mRNA was translated to GABA<sub>C</sub> receptors in the developing cortex, a Western blot analysis with the polyclonal GABA<sub>C</sub> receptor antibody rho63 (Enz et al., 1996), was carried out on protein homogenates of E18 cortices. The rho63 antibody labeled a strong single band of 60 kDa, in accordance with the expected size (Rozzo et al., 2002) of the protein (Fig. 2B). In summary, these results demonstrate that GABA<sub>C</sub> receptor mRNA and protein exist in the developing cerebral cortex.

Expression and distribution of GABA<sub>C</sub> receptor subunit mRNA and GABA<sub>C</sub> receptor protein detection in embryonic cortex. (A) CYBR-gold stained agarose gel with PCR products for rho1 (seen as a band of 633 bp) and rho2 (456 bp) subunits of GABA<sub>C</sub> receptors in organotypic neocortical slices. The beta1 GABA receptor subunit (521 bp) serves as positive control. (B) Western blot analysis of GABA<sub>C</sub> receptor protein in cortices of E17 mouse embryos and whole brain of adult mice. Homogenates are separated on a 10% gel and visualized with a polyclonal antibody identifying both GABA<sub>C</sub> subunits. Numbers at the right indicate molecular weight standards in kDa.

GABA<sub>C</sub> receptors are functional in the intermediate zone

We next addressed the question whether these GABA<sub>C</sub> receptors are of any functional significance. For this purpose we analyzed GABA induced increases in Calcium Green-1 fluorescence, since at this developmental stage GABA triggers a Ca<sup>2+</sup> influx due to depolarizing GABA responses (Owens et al., 1996). The calcium imaging experiments were performed either in the intermediate zone or in the upper part of the corticale plate.

In the intermediate zone, bath application of 100 µM GABA induced a Ca<sup>2+</sup> increase (Fig. 3A), indicating that ionotropic GABA receptors mediate depolarizing membrane responses. Bath application of the GABA<sub>C</sub> agonist CACA (100 µM, applied in the continuous presence of 100 µM BMI) induced a Ca<sup>2+</sup> increase in 132 of 192 cells (in 12 slices) that previously displayed GABA-induced Ca<sup>2+</sup> transients (Fig. 3A<sub>1</sub>). The CACA mediated increase in fluorescence ratio F/F<sub>0</sub>, amounted to 85% ± 2.5% of the GABA-induced response. The remaining 60 of these 192 cells did not show any CACA induced Ca<sup>2+</sup> increase. The GABA-induced Ca<sup>2+</sup> responses were significantly (P<0.0001) reduced in the presence of 50 µM TPMPA in the majority of the cells (n=78 of 105 cells in 18 slices, Fig. 3B). In the remaining cells the GABA induced Ca<sup>2+</sup> response was either eliminated in the presence of TPMPA (n=9 of 105 cells) or TPMPA had no influence on the GABAergic response (n=18 of 105 cells). The TPMPA mediated reduction of GABAergic Ca<sup>2+</sup> responses was reversed after TPMPA washout in all 44 cells (in six slices) investigated (Fig. 3B). Blockade of GABA<sub>A</sub> receptors with 100 µM BMI abolished the GABA induced Ca<sup>2+</sup> increase in the majority of the neurons (n=73 of 80 cells in 11 slices, Fig. 3C<sub>1</sub>).
However, in the remaining cells GABA still induced a Ca\(^{2+}\) increase, which was significantly (\(P<0.018\)) smaller as compared to the Ca\(^{2+}\) responses before BMI application (44.5\%±8.7\%, \(n=7\) cells, Fig. 3C). Co-application of 100 \(\mu\)M BMI with TPMPA abolished the GABA induced Ca\(^{2+}\) increase in all neurons (Fig. 3C). In summary, these results demonstrate that in the intermediate zone GABA\(_C\) receptors are functionally expressed and contribute to GABA responses, despite the fact that GABA\(_A\) receptors still dominate GABA responses in most neurons.

In the upper cortical plate, bath application of 100 \(\mu\)M GABA also induced a marked increase in the intracellular Ca\(^{2+}\) concentration (Fig. 4A), while bath application of 100 \(\mu\)M CACA did not induce any Ca\(^{2+}\) increase in 130 cells (of three slices) responsive to GABA (Fig. 4A). Blockade of GABA\(_C\) receptors with 50 \(\mu\)M TPMPA had no significant (\(P=0.107\)) effect on the GABA induced Ca\(^{2+}\) transients (93.8\%±4.8\% of control, \(n=29\) cells in four slices, Fig. 4B, D). In the presence of 100 \(\mu\)M BMI, applied either alone (\(n=80\) cells in 11 slices) or together with TPMPA (\(n=29\) cells in four slices), the GABA induced Ca\(^{2+}\) increase was completely abolished in all neurons (Fig. 4B–D). The complete blockade of GABA induced Ca\(^{2+}\) responses by BMI and the lack of any significant effect of TPMPA or CACA suggest that GABA\(_C\) receptors are not functional in the cortical plate.

In conclusion, these observations suggest that GABA\(_C\) receptors are functionally expressed in the intermediate zone, while GABA\(_A\) receptors are present in both the intermediate zone and the cortical plate.

**DISCUSSION**

Our results demonstrate that (i) application of either the GABA\(_A\) specific antagonist TPMPA, the subtype independent antagonist picrotoxin or co-application of TPMPA and BMI impedes migration, while the GABA\(_A\) antagonist BMI facilitates migration, that (ii) GABA\(_C\) receptors are expressed on mRNA and protein levels in the late embryonic cortex, and that (iii) only in the intermediate zone GABA\(_C\)
receptor agonists induces Ca\(^{2+}\)/H11001 responses and GABAC antagonists reduces GABAergic Ca\(^{2+}\)/H11001 transients. We conclude from these observations that GABAC receptors are expressed in the intermediate zone of the late embryonic mouse cortex and promote radial neuronal migration, while GABAA receptors impede radial migration.

Role of GABA in the regulation of radial migration

GABA receptors have been implicated in the regulation of neuronal migration for several cell types, including interneurons (López-Bendito et al., 2003; Cuzon et al., 2006), olfactory bulb neurons (Bolteus and Bordey, 2004a) and hippocampal neurons (Manent et al., 2005). In accordance with previous studies performed in rats (Behar et al., 2000; Heck et al., 2007), we also observed in organotypic neocortical slices from embryonic mice that blockade of GABA\(_{\text{A}}\) receptors enhances neuronal migration. This finding suggests that GABA\(_{\text{A}}\) receptors are implicated in the termination of migration and supports the concept that GABA is an important modulator of cortical migration (Behar et al., 1999). In a previous study, we could already demonstrate that functional inhibition of GABA\(_{\text{A}}\) receptors in vivo induced heterotopias and disturbances of cortical layer formation (Heck et al., 2007), additionally supporting the essential role of GABA\(_{\text{A}}\) receptors as a stop signal for migrating neurons (Owens and Kriegstein, 2002; Manent and Represa, 2007; Heng et al., 2007). In agreement with previous findings in rats (Behar et al., 2000), we demonstrate in the present study that picrotoxin, a blocker of both GABA\(_{\text{A}}\) and GABA\(_{\text{C}}\) receptors, massively impedes migration in mice organotypic neocortical slices. However, these previous findings do not unequivocally prove the implication of GABA\(_{\text{C}}\) receptors, as picrotoxin is, for example, also an inhibitor of homomeric glycine receptors consisting of alpha subunits (Pribilla et al., 1992). Therefore we used the specific GABA\(_{\text{C}}\) receptor blocker TPMPA, which gave nearly identical results as picrotoxin. The combined blockade of GABA\(_{\text{A}}\) and GABA\(_{\text{C}}\) receptors by co-application of TPMPA and BMI, which should resemble the inhibitory action of picrotoxin, also impedes radial migration. On the other hand, addition of the GABA\(_{\text{C}}\) receptor agonist CACA leads to an impairment of migration and thus induces the same effect as the GABA\(_{\text{C}}\) receptor antagonist. A similar apparent paradoxical effect has been also reported in vivo and in vitro for the GABA\(_{\text{A}}\) receptor agonist muscimol, which also induced similar effects as GABA\(_{\text{A}}\) receptor antagonists (Heck et al., 2007). These paradoxical effects can be explained by a desensitization of GABA receptors upon longer application of GABAergic agonists, which will lead to a functional loss of GABA\(_{\text{A}}\) receptor mediated

Fig. 4. Calcium responses to GABA and CACA application in the cortical plate. (A) Typical microfluorimetric image of Calcium Green filled neurons. Only fluorescent neurons in the upper part of the cortical plate (CP) were selected for analysis. (A2) Consecutive traces of relative Calcium Green fluorescence ratio \(\text{F/F}_0\) induced by bath application of 100 \(\mu\text{M}\) GABA or 100 \(\mu\text{M}\) CACA in three cells marked with white circles in (A1). While GABA reliably induced Ca\(^{2+}\) transient, CACA failed to induce Ca\(^{2+}\) responses in the CP. (B) Consecutive traces of GABAergic Ca\(^{2+}\) responses demonstrating that the Ca\(^{2+}\) influx was not affected after inhibition of GABA\(_{\text{A}}\) receptors by TPMPA (50 \(\mu\text{M}\)), but was completely blocked in the presence of both TPMPA and BMI. (C) The GABA\(_{\text{A}}\) receptor antagonist BMI totally blocked the GABAergic Ca\(^{2+}\) responses. (D) Statistical analysis of the relative GABAergic responses. While TPMPA \((n=29\) cells\) had no obvious effect, BMI either alone \((n=80\) cells\) or coapplied with TPMPA \((n=29\) cells\)) completely blocks Ca\(^{2+}\) transients in all cells. Columns represent mean±SEM. Wilcoxon test, *** \(P<0.001\), ns=\(P>0.05\).
responses. In summary, these findings demonstrate that GABA<sub>C</sub> receptors are indeed involved in the regulation of radial migration and that they promote the migration out of deeper cortical layers.

GABA<sub>C</sub> receptor mediated responses in the intermediate zone and cortical plate

In accordance with previous reports that demonstrate the expression of GABA<sub>C</sub> receptors in many areas of the immature brain (Rozzo et al., 2002) including the lower neocortical layers of embryonic mouse brain (Fukui et al., 2008), we detected GABA<sub>C</sub> receptor forming rho subunits in the late embryonic mouse cortex. In addition to the study of Fukui et al. (2008), we were able to identify the expression of both rho1 and rho2 subunits in the immature cortex and demonstrated that GABA<sub>C</sub> receptor protein can also be detected in the immature mouse cortex, suggesting the establishment of functional GABA<sub>C</sub> receptors. Accordingly, we provide evidence for functional GABA<sub>C</sub> receptors in our Ca<sup>2+</sup> imaging experiments. These experiments revealed that the GABA<sub>C</sub> receptor antagonist TPMPA was only effective in the intermediate zone and without effect in the upper cortical plate. In addition, the GABA<sub>A</sub> receptor antagonist BMI blocked GABAergic responses in all neurons in the CP, while in some neurons of the intermediate zone BMI insensitive GABAergic Ca<sup>2+</sup> transients were observed. It is unlikely that these remaining responses were caused by incomplete inhibition of GABA<sub>A</sub> receptors due to the competitive nature of BMI inhibition, because of the rather high BMI concentration of 100 &mu;M used in these experiments. Since GABA<sub>C</sub> receptors show less desensitization than GABA<sub>A</sub> receptors (Amin and Weiss, 1994), differences in receptor desensitization by bath applied GABA pulses can also not account for the low number of GABA<sub>C</sub> mediated responses in these experiments. And finally, the GABA<sub>C</sub> receptor agonist CACA induced Ca<sup>2+</sup> transients only in the intermediate zone and was without effect in the upper cortical plate. However, CACA-induced Ca<sup>2+</sup> transients were observed in about 70% of the neurons, while in only ~10% of the neurons GABAergic responses are either BMI insensitive or completely inhibited by TPMPA. One possible explanation for this inconsistent observation could be the expression of GABA receptors composed of a combination of rho subunits with other GABA<sub>A</sub> subunits, which resulted in GABA receptors with a mixed pharmacology (Hartmann et al., 2004; Milligan et al., 2004). Since rho subunits are involved in ligand binding, the GABA affinity of such rho subunit containing GABA receptors may also be higher than in classical GABA<sub>A</sub> receptors (Bormann, 2000). In any case, despite the fact that GABA receptors with a complex subunit composition may exist in the intermediate zone, the detection of BMI-insensitive and TPMPA-abolished GABAergic responses indicate that GABA<sub>A</sub> receptors are functional in the intermediate zone, while they are absent in the upper cortical plate. On the other hand, CACA induced weaker Ca<sup>2+</sup> responses than GABA and GABAergic Ca<sup>2+</sup> transients were only partially reduced by TPMPA, indicating that GABA<sub>A</sub> receptors still dominated the GABA responses in most neurons of the intermediate zone. Because a reliable identification of migrating neurons by their morphological appearance in our Ca<sup>2+</sup> imaging experiments was not possible, we could not discriminate whether GABA<sub>C</sub> receptors are present in a particular cellular subpopulation of the intermediate zone. Although in this layer a high number of migrating neurons is expected (Ayala et al., 2007), we cannot finally prove whether migrating neurons express GABA<sub>C</sub> receptors.

It has been previously shown that bicuculline completely blocks GABA induced currents in the ventricular zone (Owens et al., 1999), suggesting the absence of GABA<sub>C</sub> receptors in these cells. The same study reported GABA<sub>A</sub> receptor mediated currents in the cortical plate, and pinpointed the lack of data for the intermediate zone. Hence, the studies by Owens et al. (1999) and our present work show a constant functional expression of GABA<sub>A</sub> receptors throughout the entire telencephalic wall of the developing cortex. In the intermediate zone a proportion of the GABAergic responses is mediated by GABA<sub>C</sub> receptors or rho subunit containing receptors, while such GABA receptor subtypes are absent in the cortical plate.

During cortical development activation of ionotropic GABA receptors leads to chloride efflux that depolarizes the cell and consequently opens voltage-dependent Ca<sup>2+</sup> channels in neurons located in the ventricular and subventricular zone, the subplate and the cortical plate (Owens et al., 1996; Fiumelli and Woodin, 2007; Hanganu et al., 2009). Accordingly, we observed GABA induced Ca<sup>2+</sup> signals in the cortical plate and in the intermediate zone. In the intermediate zone these signals could be evoked by the selective activation of GABA<sub>C</sub> receptors, indicating that these receptors are sufficient to trigger Ca<sup>2+</sup> responses. Such Ca<sup>2+</sup> transients are an essential parameter for the induction and maintenance of radial migration (Behar et al., 1996; Komuro and Rakic, 1996; Eddy et al., 2000). Beside the Ca<sup>2+</sup> mediated effects of GABA<sub>C</sub> receptor activation on neuronal migration, we cannot rule out the possibility that activation of GABA<sub>C</sub> receptors influences radial migration independent of Ca<sup>2+</sup> fluxes. For example, the microtubule associated protein MAP1B, which participates in the regulation of radial migration (Gonzalez-Billault et al., 2005), interacts with GABA<sub>C</sub>, but not with GABA<sub>A</sub> receptors and modulates their activity (Billups et al., 2000). Such mechanisms, or a stage specific expression of Ca<sup>2+</sup> dependent regulators of cellular motility in migrating neurons, may explain why the same neurotransmitter GABA, mediating similar Ca<sup>2+</sup> responses after activation of either GABA<sub>A</sub> or GABA<sub>C</sub> receptors, can exert differential effects on radial migration.

In summary, our results provide additional evidence that GABA acting via GABA<sub>A</sub> and GABA<sub>C</sub> receptors plays an essential role in the control of neocortical radial migration. Therefore, possible implications on early cortical development should be carefully considered with any therapy interfering with the GABAergic system during fetal development (Manent et al., 2007; Henschel et al., 2008).
An updated model on the role of GABA in neuronal migration

Based on a series of experiments that indicate the important role of GABA for radial migration (Behar et al., 1994, 1996, 1998, 2000, 2001) Behar and colleagues introduced a model that integrates all of their observations (Behar et al., 2000). According to this model, GABA<sub>A</sub> receptors are mediating a stop signal required for the termination of radial migration, while GABA<sub>B</sub> receptors direct cells in the intermediate zone to enter the cortical plate, and activation of GABA<sub>C</sub> receptors is essential for the migration of neurons out of the ventricular/subventricular zone into the intermediate zone (Behar et al., 2000; Owens and Kriegstein, 2002; Manent and Represa, 2007). Our present work adds two important points to this model on the role of the GABAergic system in radial migration (Behar et al., 2000; Owens and Kriegstein, 2002; Manent and Represa, 2007; Heng et al., 2007).

Our main contribution is the direct evidence that GABA<sub>C</sub> or rho subunit containing receptors are functionally expressed in the intermediate zone of the developing neocortex. While the existence of these receptors was only proposed from the observation that picrotoxin impedes migration in vitro, the Ca<sup>2+</sup> imaging studies in the present study demonstrate that in the intermediate zone a proportion of the GABAergic responses is mediated by GABA<sub>C</sub> receptors or rho subunit containing receptors, while such GABA receptor subtypes are absent in the cortical plate. In addition, we were able to show that more specific antagonists of GABA<sub>C</sub> receptors impede migration. This observation does not only support the results of the picrotoxin experiment, but also reveals that the promigratory effect of GABA<sub>C</sub> receptors dominates the effect of GABA<sub>A</sub> receptors. GABA<sub>A</sub> receptors in the intermediate zone could not compensate a deficient activation of GABA<sub>C</sub> receptors.

GABA<sub>C</sub> receptors with their high affinity to GABA (Bormann, 2000) are perfectly located in the deeper layers of the cerebral cortex, where due to the primarily superficial localization of GABAergic neurons a low GABA concentration is assumed at late embryonic stages (Cobas et al., 1991; Owens et al., 1999; Behar et al., 2000; Haydar et al., 2000; Owens and Kriegstein, 2002). We propose that activation of GABA<sub>C</sub> receptors delivers a signal that maintains migration throughout the intermediate zone (Fig. 5), rather than being only a signal for the transition from the ventricular/subventricular zone into the intermediate zone (Behar et al., 2000). In superficial regions other receptors, like GABA<sub>B</sub> or NMDA receptors, must be implicated in the maintenance of the migratory process (Behar et al., 2000; Owens and Kriegstein, 2002; Reiprich et al., 2005; Manent and Represa, 2007).

Fig. 5. Model of GABA<sub>A</sub> and GABA<sub>C</sub> receptor-dependent radial migration in the developing cortex. Schematic illustration of the neonatal cerebral cortex with outside directed GABA gradient (grey colored gradient). In the intermediate zone (IZ) migrating neurons express functional GABA<sub>A</sub> (blue discs) and GABA<sub>C</sub> receptors (orange discs), whereas in the cortical plate (CP) migrating neurons express only functional GABA<sub>A</sub> receptors. Due to the outside directed GABA gradient the low-affinity GABA<sub>A</sub> receptors are only activated in the CP, whereas the lower GABA concentration in the IZ is sufficient to activate the high affinity GABA<sub>C</sub> receptors. Activation of GABA<sub>A</sub> receptors is necessary to support migration in the intermediate zone (GO sign), while activation of GABA<sub>C</sub> receptors contributes to termination of migration (STOP sign). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

REFERENCES


APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.neuroscience.2010.01.049.