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TRANSIENT MGLU$_5$R INHIBITION ENHANCES THE SURVIVAL OF GRANULE CELL PRECURSORS IN THE NEONATAL CEREBELLUM

C. KUBERA, A. L. HERNANDEZ, V. HENG AND A. BORDEY*

Departments of Neurosurgery, and Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06520-8082, United States

Abstract—The generation of the most abundant neurons of the cerebellum, the granule cells, relies on a balance between clonal expansion and apoptosis during the first 10 days after birth in the external germinal layer (EGL). The amino acid glutamate controls such critical phases of cell development in other systems through specific receptors such as metabotropic glutamate receptor 5 (mGlu$_5$R). However, the function of mGlu$_5$Rs on the proliferation and survival of granule cell precursors (GCPs) remains elusive. We found mGlu$_5$R mRNA transcripts in EGL using RT-PCR and observed mGlu$_5$R-mediated Ca^{2+} responses in GCPs in acute slices as early as postnatal day (P) 2–3. Using *in vivo* injections of the selective non-competitive mGlu$_5$R antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP) in P7–P9 mice, we found a 20% increase in the number of proliferative GCPs labeled at P7 with the S-phase marker bromodeoxyuridine (BrdU), but no increase in cell proliferation examined 2 h following a BrdU injection. Furthermore, similar treatments led to a significant 70% decrease in the number of apoptotic GCPs in the EGL as determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. In contrast, *in vivo* treatment with the mGlu$_5$R agonist (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG) resulted in a ~60% increase in the number of TUNEL-labeled GCPs compared to control. These findings identify a unique role for glutamate acting at mGlu$_5$Rs as a functional switch regulating GCP survival in the EGL, thus controlling the total number of cerebellar granule cells produced.

Keywords: glutamate receptor, neurogenesis, neuronal precursors, metabotropic glutamate receptor, proliferation, apoptosis.

*Corresponding author. Address: Yale University School of Medicine, 333 Cedar St., FMB 422, New Haven, CT 06520-8082, United States. Tel: +1-203-737-2515; fax: +1-203-737-2159.
E-mail address: angelique.bordey@yale.edu (A. Bordey).

Abbreviations: aCSF, artificial cerebrospinal fluid; Bok, BCL2-related ovarian killer protein; BrdU, bromodeoxyuridine; Casp14, caspase 14; CHPG, (*RS*)-2-chloro-5-hydroxyphenylglycine; DHPG, (*S*)-3,5-dihydroxyphenylglycine; EGL, external germinal layer; GCPs, granule cell precursors; icv, intracerebroventricularly; IGL, inner granular layer; IP, intraperitoneal; mGluR, metabotropic glutamate receptor; MPEP, 2-methyl-6-(phenylethynyl)pyridine; OGB, Oregon Green BAPTA AM; P, postnatal day; PBS, phosphate buffered saline; SEM, standard error of the mean; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

INTRODUCTION

The development of the postnatal cerebellum, and specifically that of the cerebellar granule cells, can be subdivided into distinct stages. Granule cell precursors (GCPs) proliferate in the outer external germinal layer (EGL) up to two weeks postnatal. Between postnatal days 6 and 9 in rodents, a percentage of the proliferating GCPs undergo apoptosis (Wood et al., 1993; Tanaka and Marunouchi, 1998). Upon cell cycle exit, GCPs migrate tangentially in the inner EGL, then turn and migrate radially to the inner granular layer (IGL) where they synaptically integrate (Altman, 1969; Hatten and Heintz, 1995; Mullen et al., 1997; Goldowitz and Hamre, 1998). Signals regulating GCP proliferation and survival are critical for controlling the ultimate number of newborn GCPs that are necessary for proper circuit formation and function.

One of these signals is the amino acid and neurotransmitter glutamate. Glutamate acting at several specific receptors controls several stages of cell development (Nguyen et al., 2001; Platel et al., 2010). One family of these receptors, the metabotropic glutamate receptor (mGluR) family, have been implicated in cell proliferation and survival in various neurogenic regions throughout the brain (Brazel et al., 2005; Di Giorgi-Gerevini et al., 2005; Castiglione et al., 2008). Of the eight mGluRs, mGlu$_4$R and mGlu$_5$R may be expressed in the EGL (Copani et al., 1998; Corti et al., 2002; Di Giorgi Gerevini et al., 2004). Indeed, injection of a mGlu$_4$R agonist *in vivo* has been shown to reduce GCP proliferation and promote differentiation in lobule V only (Canudas et al., 2004). Injections of mGlu$_5$R antisense in the neonatal cerebellum have been shown to reduce granule cell density in the IGL, but the EGL was not examined (Catania et al., 2001). Studies done in culture have conflicting outcomes as to whether mGlu$_5$R promotes cell survival or not (Copani et al., 1998; Bruno et al., 2000; Battaglia et al., 2001; Catania et al., 2001; Di Giorgi-Gerevini et al., 2005; D'Antoni et al., 2011). However, the role of mGlu$_5$Rs has not been explored in the EGL. mGlu$_5$Rs are G-protein coupled receptors leading to Ca^{2+} increases through inositol trisphosphate. Activation of these receptors is thus expected to regulate the behavior of GCPs through Ca^{2+} increases. Indeed, Ca^{2+} activity is tightly correlated with progression through the cell cycle and cell migration including the tangential migration of GCPs in the inner EGL (Komuro and Rakic, 1996, 1998; Parkash and Asotra, 2010).

Interestingly, mGlu$_5$R has been shown to be misregulated in a number of developmental disorders, including

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Fragile X Syndrome, autism, tuberous sclerosis, and attention deficit hyperactivity disorder (Boer et al., 2008; Dolen and Bear, 2008; Carlson, 2012; Elia et al., 2012). Although all of these developmental disorders are typically thought of as cortical cerebral in nature, they all have a cerebellar pathology as well (Marti-Bonmati et al., 2000; Koekkoek et al., 2005; Huber, 2006; Casanova, 2007; Fatemi et al., 2008). In addition to its role in motor control, the cerebellum is now well-known to have cognitive and affective functions (Baillieux et al., 2008). Considering that selective $mGlu_5R$ antagonists are in clinical trials for treatment of Fragile X syndrome and autism, it is important to know the effects of these drugs on the development of all brain systems, including the cerebellum.

In this study, we focused on $mGlu_5Rs$ and sought to determine its role in GCP proliferation and survival in the EGL of the postnatal murine cerebellum. We found that activation of $mGlu_5Rs$ led to Ca^{2+} increases in GCPs of the EGL. *In vivo* injection of the non-competitive $mGlu_5R$ antagonist 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) resulted in a significant decrease in the number of apoptotic GCPs in the EGL. In contrast, *in vivo* injection of the $mGlu_5R$ agonist (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG) had the opposite effect. These findings identify a unique role for $mGlu_5Rs$ in the number of apoptotic cells during postnatal cerebellar development and suggest that $mGlu_5Rs$ may be a deciding factor in determining the total number of cerebellar granule cells.

EXPERIMENTAL PROCEDURES

Animals

CD-1 mice of either gender were obtained from Charles River Laboratories (Boston, MA). All experiments were performed in accordance with Yale Animal Care and Use Committee guidelines.

RT-PCR for $mGlu_5R$ transcripts

One or 10 cells were extracted from the EGL of P7–P12 acute horizontal cerebellar slices using a pulled glass patch pipet containing a solution of water, 23 mM DTT, and 4 units μl^{-1} RNase inhibitor (Applied Biosystems, Foster City, CA). The pipet tip was then broken into a pcr tube containing reagents for reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA), and the reaction was run in a MyCycler Thermal Cycler (BioRad, Hercules, CA) according to kit instructions. Once the mRNA was converted to cDNA, 36 cycles of PCR amplification were performed using Platinum *Taq* polymerase reagents (Invitrogen, Carlsbad, CA) and primers for $mGlu_5R$: forward 5′-GTCCTTCTGTTGATCCTGTC-3′; reverse 5′-ATGCAGCATGGCCTCCACTC-3′ (product size: 216 bp). A second round of PCR (36 cycles) was performed using fresh master mix and 3 μl of sample from the first reaction. End point PCR product was resolved on a 2% agarose gel in TAE buffer.

Calcium imaging

Acute sagittal or horizontal cerebellum brain slices were prepared from P2–P4 or P7–P12 mouse pups. Mice were anesthetized with pentobarbital (50 mg kg^{-1}) via intraperitoneal (IP) injection, decapitated, and the brain was rapidly removed into ice cold oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 125; KCl 2.5; $CaCl_2$ 1.8; $MgCl_2$ 1; $NaHCO_3$ 25; glucose 10 as previously reported (Dave and Bordey, 2009). 300 μm sagittal or horizontal sections were cut using a Leica VT 1000S-vibrating microtome and placed in a recovery chamber containing oxygenated aCSF at room temperature for 30–60 min prior to loading. To load Ca^{2+} indicator dyes, slices were incubated with 5 μM Fluo-4 AM + 5 μM Oregon Green BAPTA AM (OGB) + 0.02% F-127 pluronic acid (Invitrogen, Carlsbad, CA) in aCSF for 30–45 min at 37 °C. Loaded slices were placed in a flow-through chamber and continuously perfused with oxygenated aCSF for 30 min prior to imaging to allow for de-esterification. Two to 5 min Ca^{2+}-imaging movies were taken in a single plane with an acquisition rate of 1.12 frames s^{-1} using an Olympus Fluoview 300 confocal microscope equipped with a 60× water immersion objective lens. Fifty to 100 μM (*S*)-3,5-dihydroxyphenylglycine (DHPG) or 500–1000 μM (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG) (both Tocris Bioscience, Ellisville, MO) was presented to cells of the EGL using pressure application (3 psi) for 30–120 s. Antagonists were bath applied through the perfusion system. Following movie acquisition, Ca^{2+}-imaging data were analyzed using CalSignal software developed by JC Platel (Platel et al., 2007), Microsoft Excel 2007 and Microcal Origin 6.0.

Drug injections

MPEP hydrochloride (Tocris Bioscience, Ellisville, MO) was given twice daily 8–12 h apart intraperitoneally (IP) at 15 mg kg^{-1} on postnatal days (P)7–P9. MPEP was dissolved at 1 mg ml^{-1} in phosphate-buffered saline (PBS). Mice in the control group were given an equal volume of PBS alone. For BrdU-labeling experiments, all mice received one IP injection of BrdU (50 μg g^{-1}) along with either the first or last drug injection. CHPG was given intracerebroventricularly (icv) once per day for two days beginning at P2. Pups were anesthetized on ice and 1–5 μl of 25 mM CHPG in sterile PBS with 0.02% Fast Green dye was injected into the lateral ventricle with manual pressure using a pulled glass pipet beveled to a <50 μm diameter. Mice in the control group were given an equal volume of PBS plus 0.02% Fast Green.

Immunostaining

Sample preparation and immunostaining were performed as previously described (Platel et al., 2009). Briefly, P9 mice were anesthetized with pentobarbital (50 mg kg^{-1} via IP injection) and decapitated. The brains were rapidly removed and drop-fixed in 4% paraformaldehyde overnight at 4 °C. For BrdU staining, free-floating sagittal brain sections (100 μm) were treated with 2 N HCl for 1 h at room temperature. Sections were washed with PBS and then blocked in PBS-blocking solution (PBS plus 0.1% Tween-20, 0.1% Triton X-100, 2% bovine serum albumin) for an additional hour. Primary antibodies (1:100 anti-BrdU BU1/75, Accurate Chemical & Scientific Corporation, Westbury, NY) were applied overnight at 4 °C. Sections were washed with multiple changes of PBS + 0.05% Tween-20 (PBS-T) and then incubated in secondary antibodies (1:1000 goat anti-rat Alexa Fluor 555, 1:10,000 DAPI; Invitrogen, Carlsbad, CA) for 1 h at room temperature. Following several washes sections were mounted on glass slides with Prolong Gold Antifade (Invitrogen, Carlsbad, CA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) on 50 μm-thick cryostat sections from P3 or P9 mice mounted on gelatin-coated glass slides. Slides were then incubated with 1:10,000 DAPI for 10 min and washed briefly. Coverslips were mounted with Prolong Gold Antifade. Slides were imaged on an Olympus FluoView 1000 confocal microscope.