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The NR2B subunit in NMDA receptors is functionally important during cerebellar granule cell migration

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Abstract

Migration of cerebellar granule cells (CGCs) from the external germinal cell layer (EGL) to the internal granule cell layer (IGL) within the cerebellar cortex is a crucial developmental process. Antagonists to NMDA receptors impair CGC migration significantly, but studies to determine which subunit subtypes control or affect migration have been controversial. Migrating CGCs transiently express NMDA receptor subunit subtypes NR1a plus NR2B. Grafted NR1−/− subunit knockout cells continue to migrate, indicating that the NR1 subunit is not necessary for migration. In the present study, the functional importance of the NR2B subtype in developing cerebellum was investigated using organotypic slice cultures prepared from postnatal day 8 (P8) rats. Slice cultures were labeled with bromodeoxyuridine (BrdU) during the first 20 h and then continuously treated with the NR2B-subtype-specific NMDA antagonist, ifenprodil, or the non-specific NMDA antagonist, APV, for 7 days. Cultures were incubated with fluorescently tagged anti-BrdU IgG and the percent of BrdU-labeled CGCs that migrated from the EGL to the IGL during treatment was analyzed using laser confocal microscopy. Migration into the IGL was significantly impaired by treatment with 0.5 and 1.0 μM ifenprodil. Fewer cells had migrated to the IGL in 1.0 μM ifenprodil than in 0.5 μM ifenprodil; there was no significant difference between the percent impairment caused by 1.0 μM ifenprodil and 50 μM APV. Untreated controls had few, if any, CGCs in the EGL at DIV 8. The percent of CGCs remaining in the EGL following treatment with antagonists significantly increased, indicating impairment of migration. In conclusion, the NR2B subunit appears to be necessary for CGC migration.

Keywords: Cerebellar granule cell migration; Cerebellum-development; NMDA receptor in development; NMDA receptor subunit composition; Neuronal migration; External germinal layer; Molecular layer; Internal granule cell layer

Immature cerebellar granule cells (CGC) migrate from the external germinal cell layer (EGL) to form the internal granule cell layer (IGL) [7]. However, the specific mechanisms which orchestrate CGC migration are not yet clear. This information is critical because CGCs that do not migrate to their appropriate destination usually fail to mature properly. Deficits in neuronal migration are involved in numerous pathological conditions as indicated by misplaced or heterotopic neurons in the cerebellum such as Zelweger's Syndrome, paroxysmal ataxia, and Fryns Syndrome. Clinical symptoms of malformed cerebellar cortex include deficits in psychomotor development, ataxia, and epilepsy [5].

During normal embryonic cerebellar development, the lateral rhombencephalon becomes the outermost cortical layer, known as the external germinal cell layer (EGL). The EGL consists of progenitor cells that undergo asymmetrical mitosis, generating immature CGCs. The EGL is thickest during postnatal day 8–10 (P8–10) in rats [1]. Newly formed CGCs are stationary for up to 48 h prior to tangential migration within the EGL, and subsequent radial migration through the molecular layer (ML) and Purkinje cell layer (PCL) [9]. CGC migration occurs during the second week of postnatal life in rats, peaking at P10 and 11 [7], and it occurs perinatally in humans [1]. Migration is a saltatory process; increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) are correlated with forward movement, whereas decreases in $[Ca^{2+}]_i$ may stop or even reverse migration [8]. However, continuously high levels of Ca^{2+} do not cause continuous forward movement. Transient Ca^{2+} fluxes, or Ca^{2+} oscillations, are required for CGC migration [10]. The frequency of Ca^{2+} oscillations, and therefore the

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rate of migration, is influenced by extracellular signals to receptors or channels, which then modulate $[Ca^{2+}]_i$, the secondary messenger [8]. The Ca^{2+} oscillations are known to be dependent on *N*-methyl D-aspartate (NMDA) receptors and N-type voltage-activated Ca^{2+} channels ($Ca_V2.2$) [8].

NMDA receptors in immature CGCs differ from those of mature CGCs. They are heteroligomeric membrane-bound receptors that consist of an NR1 subunit and at least one NR2 subunit. Eight variants of the NR1 (a–h) and four variants of the NR2 (A–D) subunits exist [16]. An NR3 subunit may also exist with two possible variants [4]. Migrating CGCs are unique in that they transiently express NMDA receptor subunit subtypes NR1a plus NR2B. As CGCs approach the IGL, expression of the NR2A subtype increases [15,17]. Mature CGCs predominantly express the NR2C subtype [4,12]. Subunit composition is important because functional properties such as the channel conductance and receptor deactivation time differ based on the subunit composition of the receptor. NMDA receptors containing NR2B subunits have the highest affinity for the voltage-dependent blocker, Mg^{2+}, and display slow decay kinetics [3,16]. NMDA receptors receive tonic stimulation by endogenous ligands in acutely prepared cerebellar slices, and the frequency of stimulation increases as the CGCs migrate [3,14]. The non-subunit subtype-specific NMDA receptor antagonists, MK-801 and D-2-amino-5-phosphopentanoic acid (D-APV) [7,13], significantly impair migration, but do not completely inhibit it [16]. The identity and functional role of each NR receptor subunit subtype during development is not clear.

NR1a plus NR2B receptors are likely candidates for the critical source of Ca^{2+} during migration. Grafted NR1−/− subunit knockout cells are able to migrate, indicating that the NR1 subunit is not necessary for migration [11]. On the other hand, NR2B subunits are regulated by numerous trophic factors, and possess an intracellular tail that is known to be regulated by intracellular kinases [16]. Metzger et al. [12] found that sustained expression of NR2B subunits in cerebellar slice cultures impaired CGC migration and Purkinje cell maturation. However, direct and indirect effects of the sustained expression could not be distinguished. Thus, the specific function of the NR2B-containing NMDA receptors in migrating CGCs remains unclear.

The objective of the present study was to determine whether inhibition of NR2B-containing NMDA receptors impairs CGC migration. Ifenprodil, an NR2B subunit-specific partial antagonist that binds to a polyamine regulatory site in the extracellular region of the subunit [2], was used to examine the function of the NR2B-containing receptors in CGC migration. Organotypic slice cultures of postnatal rat cerebellum were used as the model system. These cultures maintain cortical structure and cell–cell interactions that are similar to or the same as those which occur *in vivo*. This makes them an ideal model system for investigating receptor responses and Ca^{2+} regulation during migration. Pulse-chase labeling with bromodeoxyuridine (BrdU) was used to track the generation and migration of CGCs because BrdU is a marker for DNA synthesis. BrdU pulse-chase labeling involves initial marking of cerebellar granule cells with BrdU, then tracking the cells in a BrdU-free medium that contains the pharmacological probe. The results indicate that the NR2B subunit is critical for CGC migration.

All animal procedures were in compliance with National Institutes of Health of the USA guidelines on animal use and care. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University. Organotypic cultures were prepared using methods described previously [6,7]. Sagittal slices of cerebellum were prepared from male and female 8–9-day-old (P8–9) Charles River rat pups (Wilmington, MA). Slices were cut approximately 400 μm thick, and remained viable as assessed by calcein-AM and ethidium homodimer-1 (Molecular Probes, Eugene, OR) staining for over 8 days. Sagittal slices through the vermis were obtained to view all cerebellar cortical layers and to avoid monitoring cells migrating out of the section plane. Slices were cultured on porous, collagen-coated membranes (Transwell, Corning, Inc., Corning, NY) suspended in culture medium (Neurobasal medium supplemented with N-2 and B-27 (Invitrogen-Gibco, Carlsbad, CA), 90 U/ml penicillin, streptomycin, and gentamicin (Sigma–Aldrich, St. Louis, MO) in 12-well culture plates (Corning, Inc.), with one slice per well. Cultures were incubated in BrdU (Molecular Probes) at 37 °C in CO_2/O_2 for 20 h. To avoid the confounding effects of NMDA receptor block on proliferation of CGCs, ifenprodil or APV were not introduced until after BrdU marking.

Following ifenprodil, incubation in BrdU, the medium was replaced with BrdU-free medium containing an NR2B-specific antagonist or the non-specific antagonist D-APV (Sigma–Aldrich). Incubation was continued for 7 days in culture medium. It contained 0.5 μM ifenprodil, 1.0 μM ifenprodil, 50 μM D-APV, or vehicle (control). Half the medium was removed and replaced with fresh medium daily. On day 8, cultures were fixed in 4% (w/v) formaldehyde ammonium bromide and incubated overnight in 1:1000 AlexaFluor 546-tagged anti-BrdU rabbit IgG (Molecular Probes). Slice cultures were mounted in Slow Fade® Light Antifade (Molecular Probes) on microscope slides and were examined using a Leica TSL laser confocal microscope (Leica Microsystems Inc., Bannockburn, IL). Fluorescence throughout the entire thickness of a 40× visual field was recorded using z-series stacks.

The number of BrdU-labeled cells in each of the EGL, ML, and IGL was counted, and the percentage in terms of the total number of cells present in the viewed section of cortex was calculated. Treatment effects were tested using a one-way analysis of variance and the Tukey–Kramer post-test for multiple comparisons. Statistical significance was set at $p < 0.05$.

The viability of cultures under control conditions as assessed by calcein-AM and ethidium homodimer-1 was 95% ± 1.7 (mean ± S.E.M.) at 3 DIV and 91.8% ± 1.0 at 8 DIV. In the control cultures, virtually all cells had migrated out of the EGL by 8 DIV and ∼60% had migrated into the IGL, as reported by Komuro and Rakic [7]. With increasing time, a greater percent of CGCs, migrated out of the EGL (results not shown). The total number of BrdU-labelled CGCs are shown in Table 1, and was not significantly different among treatment groups. In contrast, migration was impaired in cultures treated with ifenprodil or D-APV (positive controls) (as shown for the representative