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Effects of elevated magnesium and substrate on neuronal numbers and neurite outgrowth of neural stem/progenitor cells in vitro

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ABSTRACT

Because a potential treatment for brain injuries could be elevating magnesium ions (Mg^{2+}) intracerebrally, we characterized the effects of elevating external Mg^{2+} in cultures of neonatal murine brain-derived neural stem/progenitor cells (NSCs). Using a crystal violet assay, which avoids interference of Mg^{2+} in the assay, it was determined that substrate influenced Mg^{2+} effects on cell numbers. On uncoated plastic, elevating Mg^{2+} levels to between 2.5 and 10 mM above basal increased NSC numbers, and at higher concentrations numbers decreased to control or lower levels. Similar biphasic curves were observed with different plating densities, treatment durations and length of time in culture. With cells were plated on laminin-coated plastic, NSC numbers were higher even in basal medium and no further effects were observed with Mg^{2+}. NSC differentiation into neurons was not altered by either substrate or Mg^{2+} supplementation. Some parameters of neurite outgrowth were increased by elevated Mg^{2+} when NSCs differentiated into neurons on uncoated plastic. Differentiation on laminin resulted in increased neurites even in basal medium and no further effects were seen when Mg^{2+} was elevated. This system can now be used to study the multiple mechanisms by which Mg^{2+} influences neuronal biology.

1. Introduction

Following brain injury due to either trauma (TBI) or hemorrhagic stroke, extracellular magnesium ions (Mg^{2+}) are depleted in brain tissue and cerebrospinal fluid, which can exacerbate harmful secondary biochemical cascades (Ginsberg, 2008; Hoane, 2004; Ovbiagele et al., 2003; Vink et al., 2009). It was found that normalizing tissue Mg^{2+} levels after injury by systemic injection significantly improved neurological outcome in animals (Sen and Gulati, 2010). Mg^{2+} levels affect hundreds of enzymes and physiological processes. The ameliorative effect of replenishing levels after brain injury has been attributed to a variety of mechanisms including reduced excitotoxicity (by blockage of *N*-methyl-D-aspartate (NMDA) glutamate receptors and calcium ion channels), reduced vasospasm, reduced levels of free radicals, and alleviation of oxidative stress, among others (Cernak et al., 2000; Sen and Gulati, 2010; van den Heuvel and Vink, 2004; Vink et al., 2009). However, in human clinical trials, the effects have been variable and primarily discouraging, although there is at least one ongoing Phase III clinical trial, which is investigating Mg^{2+} solution injections immediately after stroke (Saver, 2010; Sen and Gulati, 2010). It has been speculated that one complicating issue for human trials is that the human brain appears to maintain brain tissue homeostasis more efficiently than animals, so that Mg^{2+} levels at the injury site may not have risen to beneficial status (Sen and Gulati, 2010). An alternative approach would therefore be to deliver Mg^{2+} solutions directly to the brain injury site. Thus, it is imperative to characterize how neural cells respond to externally elevated Mg^{2+}.

Abbreviations: AAA, triple antibiotic/antimycotic; ATCC, American Type Culture Collection; CV, crystal violet; DAPI, 4′,6-diamidino-2-phenylindole; DMEM/F12, 50:50 mixture of Dulbecco's Modified Eagle's medium and F-12 culture medium; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; FCS, fetal calf serum; FGF-2, fibroblast growth factor 2; GFAP, glial fibrillary acidic protein; IACUC, Institutional Animal Care and Use Committee; NMDA, *N*-methyl-D-aspartate; NSC, neural stem/progenitor cell; NSDM, neurosphere differentiation medium; NSGM, neurosphere growth medium; NST, neuron specific (type III beta isoform) tubulin; PD(s), plating density(ies); RFU, relative fluorescence units; TBI, traumatic brain injury; TTX, tetrodotoxin.

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We studied the effects of increasing external Mg^{2+} in "neurosphere" cultures of neonatal murine brain-derived neural stem/progenitor cells (NSCs). The effects of Mg^{2+} on primary cultured neurons have been examined extensively. In early years, this lead to the determination that Mg^{2+} acts as a voltage dependent gate for *N*-methyl-D-aspartate (NMDA) glutamate receptor (Nowak et al., 1984). While some have reported toxicity due to elevated Mg^{2+} (Dribben et al., 2010), others have described beneficial effects in culture, i.e., increased synaptic plasticity (Slutsky et al., 2004) and protection of dopaminergic neurons in culture (Hashimoto et al., 2008). Removal of Mg^{2+} from cultured neurons is routinely used as a cell culture model of epilepsy (Tancredi et al., 1990). But there is still work needed to understand all of the mechanisms of how Mg^{2+} influences neurons, since this ion is a co-factor in over 300 enzymes in the cell. Cultures of purified NSCs, were first characterized in the early 1990s (Reynolds et al., 1992). The benefits of this culture system are that they are primary neural cells, i.e., they do not form tumors when implanted into an animal, as would cells derived from a cancer (Vescovi et al., 2006). Therefore, their biochemistry, both before and after differentiation into neurons and glia, is considered to be characteristic of primary brain cells. The NSC cultures provide advantages over non-neurosphere forming primary brain cell cultures because they are more homogeneous, i.e., they have a greatly reduced contamination with cells from the meninges and blood vessels. During the time periods that we tested in this study, the cultures were primarily composed of undifferentiated NSCs (immunostained with anti-nestin), including both true pluripotent stem cells and more restricted potential progenitor cells (Pastrana et al., 2011) and, even before differentiation, they contain subgroups of cells that are maturing into astrocytes, oligodendrocytes and neurons (Struckhoff and Del Valle, 2013). These cultures allowed us to study effects on both the stem/progenitor cells and then neuronal cells that differentiate from them and begin expression of neuron-specific markers. We used these cultures to explore the effects of Mg^{2+} on NSC cell number, differentiation into neurons and neurite outgrowth, on two substrates, which had different effects on NSC growth.

2. Materials and methods

"Neurosphere" cell cultures (floating cell clusters composed mostly of NSCs) were prepared from newborn (P5–P8) C57/Bl-6 mice (6–8 mice per preparation) via published methods (Gage, 2000; Johansson et al., 1999). All animal procedures were approved by the University of Cincinnati IACUC and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the NIH. Briefly, cerebral cortices (plus hippocampi) were disaggregated with papain for 30 min at room temperature (RT) (16 U/mL papain, 2 mM EDTA, 1 mM cysteine in Hank's balanced salt solution with antibiotics (HBSS+), pre-activated at 37 °C for 30 min) and enzyme action was terminated with soybean trypsin inhibitor (1.25 mg/mL; Sigma, St. Louis, MO). After mechanical disaggregation (passage through 18, 21, and 23 gauge needles) and centrifugation (10 min, 377 × *g*), cells were centrifuged again in sucrose (0.9 M in HBSS+, 15 min) to remove myelin and debris. Cells were plated (1.5×10^6 cells/uncoated T75 flask) in "neurosphere growth medium" (NSGM: DMEM/F12 (Sigma–Aldrich, St. Louis, MO) with 2% B27 supplements (Life Technologies, Grand Island, NY), 2 mM L-glutamine, 1× triple antibiotic/antimycotic solution (AAA, HyClone, UT), 27.5 μM β-mercaptoethanol (Invitrogen, Carlsbad, CA), 20 ng/mL epidermal growth factor (EGF) and 10 ng/mL fibroblast growth factor (FGF-2) (both from Sigma)). Cells were incubated (5% CO_2, 100% humidity, 37 °C), fed partial medium changes (2–3 days) and numerous floating neurospheres were evident by 1 week. These methods are standard in the field and the neurospheres were comprised of primarily cells that were positive for antibody staining for nestin and glial fibrillary acidic protein (GFAP), with scattered mature neurons and oligodendrocytes (not shown), as has been described in other publications (Louis et al., 2013; Struckhoff and Del Valle, 2013). Thereafter, neurospheres were passaged 1–2 times/week (treated with trypsin/EDTA (1× manufacturer's recommendations) for 15 min, followed by centrifugation and resuspension in NSGM and replating at the original density) and used between 2 and 10 passages. For experiments, the neurospheres were dissociated with trypsin/EDTA (as for passaging), filtered (70 μm pore size) and plated in half the final volume of a 'neurosphere differentiation medium' (NSDM: NSGM minus EGF and FGF-2, with 1% fetal calf serum (FCS) (Fisher-HyClone, Defined)) (see results for plating densities). Cells that were plated on uncoated plastic were able to attach to the plastic, which is in agreement with previous studies that have shown that mouse, but not rat, NCSs can attach to uncoated culture plastic without requiring an additional substrate protein (Ray and Gage, 2006). Mg^{2+} compounds were diluted in NSDM and added to these dissociated cultures at 24 h after plating (final volumes: 200 μL/96 well, 1 mL/24 well), and cells were fed by half-well medium changes every 2–3 days. For laminin coating, wells were pre-treated (2 h) with NSDM or 20 μg/mL laminin (Sigma) in NSDM in 1/2 the final volume, before adding cells as above. For each Mg^{2+} concentration tested, from three to six wells were treated per experiment (one experiment equaled one litter of pups used). Values for each condition were averaged per experiment and experiments were repeated at least three times, so *n* values were at least three per condition. Materials not described were from Thermo-Fisher (Pittsburgh, PA).

For the alamar blue (resazurin dye) assay, dissociated NSCs or human liposarcoma cells (SW872 cells, ATCC: HTB-92, maintained in DMEM/F12 with 10% FBS and Primocin (antibiotic solution, Invitrogen)) were plated and treated in 96-well plates (BD Falcon Microtest™ Optilux™, Belford, MA) for 2 days. Then 200 μL of sterile-filtered, 0.5 mg/mL resazurin sodium salt (Sigma) in phosphate buffered saline (PBS: pH 7.4) was added per well and incubated at 37 °c for 1 h. Fluorescence (λ_{ex} 530 nm, λ_{em} 590 nm) was measured with a Spectra max Gemini XPS plate reader (Molecular Devices, Sunnyvale, CA). Controls (solutions in cell-free wells) were treated the same way.

For the crystal violet (CV) assay, cells were plated and treated in standard cell culture 96 well plates, fixed at appropriate times (RT, 10 min in 2% glutaraldehyde in PBS, then 10 min in 4% glutaraldehyde), rinsed, stained with CV (Allied Chem. Corp., NY, NY, 0.1% in water, 30 min), rinsed in distilled water and air-dried. The color was extracted with 10% acetic acid in water and staining intensity was read (λ_{em} 540 nm) in an EL800 plate reader (Bio-Tek Instruments, Winooski, VT) and normalized to averaged controls per experiment.

For immunostaining, cells were plated on autoclaved glass coverslips (12 mm) in 24 well plates at 39 K/cm^2. These were treated (as described below), fixed (RT, 20 min in 4% paraformaldehyde) and immunostained for neurons and nuclear staining via standard methods (blocking with 10% FCS and 0.2% Triton X-100 for 1 h, overnight primary antibody incubation at room temperature, rinses with PBS, 2 h secondary antibody incubation, rinses with PBS). The primary antibody was neuron-specific tubulin (NST; mouse anti-type III β tubulin monoclonal antibody, Sigma, 1:1000 dilution) and the secondary was Alexa 488 donkey anti-mouse (Life Technologies, 1:1000) with addition of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, 1:1000) to stain nuclei. Coverslips were mounted on slides using Fluoromount (Southern Biotech, Birmingham, AL).

Micrographs of CV-stained cells were taken on an Olympus IMT-2 (Olympus, Japan) inverted microscope with a QICam cooled CCD camera (Q Imaging, Canada). Micrographs of immunostained or DAPI stained cells (grown on glass coverslips, then mounted on

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