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EXCITATORY TONUS IS REQUIRED FOR THE SURVIVAL OF GRANULE CELL PRECURSORS DURING POSTNATAL DEVELOPMENT WITHIN THE CEREBELLUM

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Abstract—In addition to protective effects within the adult central nervous system (CNS), *in vivo* application of *N*-methyl-D-aspartate inhibitors such as (+) MK-801 have been shown to induce neurodegeneration in neonatal rats over a specific developmental period. We have systematically mapped the nature and extent of MK-801-induced neurodegeneration throughout the neonatal murine brain in order to genetically dissect the mechanism of these effects. Highest levels of MK-801-induced neurodegeneration are seen in the cerebellar external germinal layer; while mature neurons of the internal granule layer are unaffected by MK-801 treatment. Examination of external germinal layer neurons by electron microscopy, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) and bromodeoxyuridine (BrdU) labeling, and caspase-3 activation demonstrate that these neurons die through the process of programmed cell death soon after they exit from the cell cycle. Significantly, ablation of caspase-3 activity completely inhibited the MK-801-induced (and developmental) programmed cell death of external germinal layer neurons. Similar to caspase-3, inactivation of muscarinic acetylcholine receptors *in vivo* using scopolamine inhibited MK-801-induced programmed cell death. By contrast, the GABAergic agonist diazepam, either alone or in combination with MK-801, enhanced programmed cell death within external germinal layer neurons. These data demonstrate that, *in vivo*, cerebellar granule neurons undergo a dramatic change in intracellular signaling in response to molecules present in the local cellular milieu during their first 24 h following exit from the cell cycle.

Key words: MK-801, diazepam, scopolamine, apoptosis, granule neurons, caspase-3.

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Abbreviations: ANOVA, analysis of variance; BrdU, bromodeoxyuridine; CNS, central nervous system; DAB, 3,3-diaminobenzidine; E, embryonic day; EGL, external germinal layer; EM, electron microscopy; GIRK, G-protein sensitive inwardly rectifying potassium channel; GPBS, 5% goat serum in PBS; IGL, internal granule layer; NMDA, *N*-methyl-D-aspartate; P, postnatal day; PCD, programmed cell death; RT, room temperature; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

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Programmed cell death (PCD) is an evolutionarily conserved process of cellular suicide (Yuan, 2006) which plays a key role in normal development (Baehrecke, 2002), and in acute and chronic neuronal loss following injury to the mammalian central nervous system (CNS) (Mattson, 2000; Hara and Snyder, 2007). One well-recognized form of neurodegenerative PCD within the adult CNS arises following excessive activation of glutamate receptors, resulting in elevated calcium influx and downstream caspase activation (Nicholls and Ward, 2000; Mattson, 2003). Blockade of *N*-methyl-D-aspartate (NMDA) receptors *in vivo* using antagonists such as the non-competitive, open-channel blocker MK-801, has been shown to confer neuroprotection from several forms of glutamate-related injury (McIntosh et al., 1989; Greensmith et al., 1994; Nicholls, 2004). In contrast, blockade of NMDA receptors during the early postnatal period is associated with a developmental vulnerability to neurodegeneration (Ikonomidou et al., 1999). This developmental window (postnatal days (P)3–P7 in rodents) coincides with an enhanced period of synaptogenesis and brain growth (Gottlieb et al., 1977; Dobbing and Sands, 1979; Hahn et al., 1983). This effect is of interest clinically, as drugs which inhibit NMDA receptors such as ethanol, phencyclidine, and anesthetics like ketamine and nitrous oxide, could potentially induce neuronal damage if fetal exposure to a sufficient dosage occurs during the critical developmental window (Bayer et al., 1993; Ikonomidou et al., 2000). Previous studies have demonstrated that these agents affect several levels of the developing CNS (Ikonomidou et al., 2000). Within the cerebellum, short term *in vitro* inhibition of NMDA signaling disrupts the migration of immature granule cells (Komuro and Rakic, 1993); however, the mechanistic consequence of sustained periods of NMDA receptor inactivation on the developing cerebellum has not been well characterized *in vivo*.

The cerebellum is principally composed of five neuronal subtypes: Purkinje and granule neurons, together with basket, stellate and Golgi interneurons. Granule cells arising from progenitors initially develop within the external germinal layer (EGL) prior to birth (Goldowitz and Hamre, 1998; Middleton and Strick, 1998; Carletti and Rossi, 2008). Following their last mitotic division, immature granule neurons migrate along Bergmann glia, through the Purkinje cell layer, to their final location within the internal granule layer (IGL). Analysis of the EGL reveals that, along with cell proliferation, a substantial amount of PCD occurs during normal development in rodents and humans (Tanaka and Marunouchi, 1998; Abraham et al., 2001). However, little is known regarding the mechanism of these effects.

Here we demonstrate that MK-801-induced death within the neonatal EGL occurs via PCD; and caspase-3 is both necessary and sufficient to regulate this process. During normal development, a significant fraction of PCD within the EGL (but not the IGL) requires caspase-3 activity; despite detectable activation of caspase-3 at both sites. Analysis of coincident bromodeoxyuridine (BrdU)/terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) labeling following MK-801 treatment indicates that a sizable fraction of granule cells undergoing PCD within the EGL, do so proximal to their last mitotic division. Finally, *in vivo* studies using scopolamine and diazepam suggest that sub-threshold levels of neuronal responsiveness may initiate the process of PCD in immature granule neurons of the EGL.

EXPERIMENTAL PROCEDURES

Animals and drug treatment

Seven-day-old *Casp3*$^{-}$/$^{-}$ mice were obtained from timed mating of our caspase-3 stock (heterozygous/heterozygous and heterozygous/homozygous intercrosses), and genotyped by PCR analysis as previously described (Woo et al., 1998). Mice were housed in a gnotobiotic facility at the Ontario Cancer Institute (Toronto, Canada). All procedures were in accordance with the Canadian Council on Animal Care (Guide to the Care and Use of Experimental Animals, Vol. 1, 2nd Ed., 1993) and the Animals for Research Act (Ontario, Canada, revised 1990), and approved by the University of Toronto Faculty Advisory Committee on Animal Services. All efforts were made to minimize the number of animals used and their suffering. For the studies described, *Casp3*$^{-}$/$^{-}$ mutants and littermate controls were examined on both inbred (C57BL/6J) and outbred (CD1) backgrounds. For the analyses shown, no significant differences in response to MK-801 was observed between *Casp3*$^{+/+}$ and *Casp3*$^{+}$/$^{-}$ littermates on either inbred or outbred backgrounds. For initial MK-801 studies in wild-type animals, 129Sv/IMJ strain mice were also examined. P7 mice were injected s.c. with either saline or MK-801 [(+) MK-801, 5 mg/kg body weight, Research Biochemicals International (RBI), Natick, MA, USA] alone, scopolamine (0.3 mg/kg, RBI), diazepam (10 mg/kg, RBI), or a combination of these drugs. S.c. injections were given at t=0, 8, and 16 h with animals killed at t=24 h. Brains were removed and fixed in 4% paraformaldehyde in 0.1 M PBS at 4 °C overnight, while tail samples were collected for genotyping. The cerebella were subsequently dissected, and either embedded in paraffin, or processed for frozen sections (caspase-3 immunohistochemistry) at 15–30 μm. Paraffin samples were cut at a thickness of 7 μm in serial sets at intervals of 150 μm.

Electron microscopy (EM)

Cerebellar samples for EM were fixed in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), at 4 °C for 12 h. Samples were subsequently impregnated with 1% osmium tetroxide, and 2% uranyl acetate in 0.1 M PBS for 1 h, then dehydrated in a series of water/ethanol and ethanol/propylene oxide baths prior to embedding in Spurr resin. Seventy nanometer ultrathin sections were then obtained and placed onto formvar-coated grids for examination on a Phillips CM 100 electron microscope equipped with a Kodak QRS 1050 digital camera.

For EM blocks, thin (1 μm) sections were also obtained and stained with Toluene Blue for analysis by light microscopy in order to aid in the positional orientation of EM photos.

Immunohistochemical analyses

Paraffin sections were de-waxed and TUNEL positive cells were identified using the TUNEL assay (FITC-TUNEL cell death assay kit, Roche Biochemicals, Indianapolis, IN, USA), in accordance with the manufacturer's instructions. For each set of tissue sections, one positive and two negative control slides were processed with each batch to verify the fidelity of TUNEL staining. Positive TUNEL controls consisted of sections taken from gamma-irradiated (2 Gy) E13.0 embryos (TUNEL-positive cells: cortical neuro-epithelium). Negative controls consisted of sections from non-irradiated E13.0 embryos and an irradiated embryo slide in which terminal deoxynucleotidyl transferase (TdT) had been eliminated from the TUNEL reaction mixture. For analysis of activated caspase-3 (New England Biolabs (NEB), Ipswitch, MA, USA, 1:200), tissues were cryoprotected in sucrose overnight at 4 °C then embedded in OCT the next day. Samples were sectioned at 15 or 30 μm on a Leitz model CM3050 cryostat. For peroxide-based immunohistochemistry of tissue sections, endogenous peroxide activity was first quenched through exposure to a freshly prepared solution of 3% H_2O_2 in 100% methanol for 30 min at room temperature (RT). Samples were then blocked in 5% goat serum, 0.2% Tween-20 in 0.1 M PBS (pH 7.4) for 1 h prior to overnight incubation in primary antibody at 4 °C. Sections were then washed three times 5 minutes and incubated in biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA, 1:200) for 2 h at RT, followed by washing and incubation with streptavidin HRP (Vector Laboratories) at 1:100 for 1 h at RT. Sections were then visualized with 3,3-diaminobenzidine with nickel enhancement (DAB, Vector Laboratories). For dual visualization of TUNEL and activated caspase-3, microwave/citrate buffer antigen retrieval was first performed on dewaxed serial paraffin sections. The sections were allowed to cool for 30 min in PBS, then blocked (as described above) for 1 h, and incubated overnight at 4 °C in anti-activated caspase-3 (NEB, 1:100) antibody. Sections were then washed three times 5 min and incubated in goat anti-rabbit Alexa 546 (Molecular Probes, Carlsbad, CA, USA, 1:200) for 2 h at RT. After washing, sections were then visualized for immunofluorescence. No significant difference was observed in the number of neurons demonstrating immunoreactivity for activated caspase-3 on paraffin sections in comparison to frozen sections. The sections were then digested with 10 mg/mL proteinase K for 15 min, followed by TUNEL as per the manufacturer's protocol. Note that proteinase K digestion (required for TUNEL) prior to this point resulted in damage to the epitope recognized by the antisera directed against activated caspase-3. No significant difference in the number of TUNEL positive cells was observed between sections which received antigen retrieval, and those that did not.

BrdU labeling

A single injection of BrdU (100 mg/kg, Sigma Aldrich, Oakville, ON, Canada) was given s.c. to P7 mice at t=12 or t=22 or t=23 h following MK-801 injection (t=0, 8, 16 h, mice killed at t=24 h). Sets of 7 μm paraffin sections were then obtained through the cerebellum in the sagittal plane at intervals of 150 μm through the central third of the cerebellum (distance covered: 1050 μm to either side of the cerebellar midline for each animal). For immunohistochemistry, sections were de-waxed, and endogenous peroxidase activity quenched as described above. Samples were incubated with 0.01% pepsin (Sigma Aldrich) in 0.01 N HCl for 15 min at 37 °C and denatured in 2 N HCl for 45 min. Sections were then neutralized in a solution of 0.1 M sodium borate (pH 8.5). After washing, slides were incubated in a solution of 5% goat serum in PBS (GPBS) for 30 min at RT. This was followed by incubation in a 1:30 dilution of mouse monoclonal anti-BrdU (Becton-Dickinson, Mississauga, ON, Canada, 347580) in GPBS overnight at 4 °C in a humidified chamber. The following day, slides were processed for DAB visualization as described above.