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Dimethyl Sulfoxide (DMSO) Produces Widespread Apoptosis in the Developing Central Nervous System

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Abstract

Dimethyl sulfoxide (DMSO) is a solvent that is routinely used as a cryopreservative in allogeneic bone marrow and organ transplantation. We exposed C57Bl/6 mice of varying postnatal ages (P0–P30) to DMSO in order to study whether DMSO could produce apoptotic degeneration in the developing CNS. DMSO produced widespread apoptosis in the developing mouse brain at all ages tested. Damage was greatest at P7. Significant elevations above the background rate of apoptosis occurred at the lowest dose tested, 0.3 ml/kg. In an *in vitro* rat hippocampal culture preparation, DMSO produced neuronal loss at concentrations of 0.5% and 1.0%. The ability of DMSO to damage neurons in dissociated cultures indicates that the toxicity likely results from a direct cellular effect. Because children, who undergo bone marrow transplantation, are routinely exposed to DMSO at doses higher than 0.3 ml/kg, there is concern that DMSO might be producing similar damage in human children.

Keywords

Brain; Development; Solvent; Degeneration; Bone Marrow Transplantation; Apoptosis; Cryopreservative; DMSO

INTRODUCTION

Apoptosis, or programmed cell death, is the natural process whereby unneeded cells are eliminated via a sequence of genetically controlled steps (Lockshin and Williams, 1964). In the brain there are two recognized periods during which cells undergo apoptosis. The first period of apoptosis occurs during the period of development when neural progenitor cells are actively proliferating (Miller et al., 2000). During this period glucocorticoids produce apoptosis in the neural progenitor cells of the external granule cell layer of the cerebellum (Noguchi et al., 2008). The second period occurs when post-mitotic neurons are integrating into functional circuits. Post-mitotic neurons that are unable to integrate successfully into developing CNS circuits and establish functional synapses are removed by apoptosis. Several classes of agents that suppress neuronal excitability can cause widespread apoptosis of neurons during this period of rapid synaptogenesis (Olney et al., 2002b). These classes include antagonists of the

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NMDA subtype of glutamate receptor (Dribben et al., 2008; Ikonomidou et al., 2000; Ikonomidou et al., 1999; Scallet et al., 2004; Wang and Johnson, 2007), GABAergic agents (Bittigau et al., 2002; Cattano et al., 2008; Ikonomidou et al., 2000; Johnson et al., 2008; Ma et al., 2007) and anticonvulsants (Bittigau et al., 2002; Glier et al., 2004; Ikonomidou et al., 2007). While the period of vulnerability in rodents is entirely post-natal, encompassing the first two weeks of post-natal life, in humans, the corresponding period of vulnerability would extend from the last trimester of *in utero* life to the first several years of post-natal life (Dobbing and Sands, 1979). Humans are commonly exposed to these classes of agents during the practice of medicine. Because exposure to these agents can produce long-term effects on cognition and behavior (Jevtovic-Todorovic et al., 2003; Noguchi et al., 2008; Wang et al., 2001; Wozniak et al., 2004; Yuede et al., 2006; Yuede et al., 2005) this drug-induced apoptosis in the developing CNS is of growing concern.

Recognition that non-human primates may also be sensitive to this drug-induced phenomenon (Farber et al., 2005; Slikker et al., 2007) has further heightened concern that human fetuses and infants, who are exposed to these agents in the practice of pediatric or obstetrical medicine or in the setting of maternal drug abuse, could be experiencing previously unrecognized neuronal injury.

While conducting several of the above studies we found evidence that dimethyl sulfoxide (DMSO), a commercially available solvent that is used extensively in industry and biological research, can also produce widespread apoptotic neurodegeneration in the developing mouse CNS. DMSO is considered a relatively safe solvent (Class 3) in doses up to 50 mg per day. It has been touted to have a wide variety of beneficial medical effects (Muir, 1996; Santos et al., 2003) and can be obtained over the Internet (*e.g.* www.dmsso.org) and used without a physician's prescription even in the absence of any compelling efficacy data. In medicine, DMSO is used routinely as a cryopreservative in allogous bone marrow and organ transplants. To help define potential risks to the developing CNS, we characterized the effects of DMSO in postnatal rodents.

MATERIALS AND METHODS

Animals and drugs

In vivo experiments were conducted with C57BL/6 mouse pups (Harlan, Indianapolis) of several different ages P0–P30. *In vitro* experiments were conducted with Sprague-Dawley rats (Harlan, Indianapolis). All animal care procedures were in accordance with standards approved by the Washington University Animal Studies Committee. For the *in vivo* drug exposure studies, mouse pups were injected with various amounts of DMSO (0.3–10 ml/kg; 99.9% ACS, spectrophotometric grade; Sigma-Aldrich, St. Louis, MO), phencyclidine (50 mg/kg; National Institute of Drug Abuse), or 10 ml/kg body weight of saline intraperitoneally. Treatment conditions were distributed equally between genders and in pre-weaning animals across litters in order to control for potential litter or gender effects. After a predetermined survival period animals were deeply anesthetized with pentobarbital and perfused via the left cardiac ventricle and ascending aorta with a fixative composed of 4% paraformaldehyde and Tris buffer, pH 7.4 (for activated caspase 3 immunohistochemistry), 4% paraformaldehyde in cacodylate buffer (for De Olmos silver staining), or 1.5% glutaraldehyde and 1% paraformaldehyde in 1.2 M pyrophosphate buffer, pH 7.4 (for plastic sections). These brains were then processed as described below.

Activated Caspase-3 Immunohistochemistry

The brains were sliced transversely on a vibratome into 75 μ m thick sections. Sections were washed in 0.01 M PBS, quenched for 10 minutes in a solution of methanol containing 3%

hydrogen peroxide, then incubated for 1 hour in blocking solution (2% BSA, 0.2% milk, 0.1% Triton X-100 in PBS), followed by incubation overnight in rabbit anti-activated caspase-3 antiserum (D175, Cell Signalling Technology: Beverly, MA) diluted 1:1500 in blocking solution. Then, the sections were incubated for one hour in biotinylated secondary antibody (goat anti-rabbit 1:200 in blocking solution), and reacted in the dark with ABC reagents (standard Vectastain ABC Elite Kit, Vector Labs; Burlingame, CA). Deposition was visualized using Very Intense Purple (VIP, Vector Labs Vector VIP SK-4600 Peroxidase Substrate Kit). Staining was visualized with light microscopy. Depending on the age of the animal, areas of damage were identified with the aid of either an atlas by Paxinos and colleagues (P0–P10 animals; [Paxinos et al., 2007]) or Hof and colleagues (P17, P30; [Hof et al., 2000]).

De Olmos silver staining

The brains were sliced transversely on a vibratome into 75 μ m thick sections. Sections of interest were chosen and processed using the following method: washed in distilled water, heated to 33°C in pre-incubation cupric-silver solution, washed in acetone for 30 seconds, incubated in silver diamine solution for 35 minutes, reduced in formaldehyde/citric acid solution for 5 minutes, bleached with 0.3% $K_3Fe(CN)_6$ for 2 minutes, stabilized in $Na_2S_2O_3$ for 1 minute, dehydrated in a series of ethanols and then cleared in xylene prior to cover slipping.

Nissl Staining

75 μ m thick sections were mounted onto gelatin coated slides and rinsed in distilled water for 10–15 minutes. Sections were stained in a 0.25% Cresyl Violet solution for 10 minutes, washed with distilled water, dehydrated in graded ethanols, and then cleared in citrisolv.

Plastic sections for electron microscopy

The brains were sliced transversely into 1 mm thick sections. Sections were incubated overnight in 1% osmium tetroxide, dehydrated in graded ethanols, cleared in toluene and embedded flat in araldite. Semithin sections, 1 μ m thick, were cut with glass knives using a MT-2B Sorval ultratome and stained with azure II and methylene blue for initial evaluation by light microscopy to identify areas with large amounts of degeneration. For electron microscopy, areas of special interest from a given block were trimmed to a smaller size, ultrathin sections were cut and suspended over a formvar coated slot grid 1×2 mm opening and stained with uranyl acetate and lead citrate and viewed in a JEOL 100 CX transmission electron microscope.

Semi-quantification of apoptotic degeneration in different brain regions

Semi-quantitative degeneration scores were used to evaluate the severity of DMSO-induced apoptosis in different regions of the brain. A single rater, blind to litter, gender, and treatment, examined the brain for activated caspase-3 immunoreactive profiles, selected a 40x field from the area of greatest damage in the region of interest, and semi-quantitatively evaluated the extent of degeneration based on the following scale: 0 = 0 to 4 caspase-3 positive cells per 40x field, 1 = 5–9 caspase-3 positive cells per 40x field, 2 = 10–14 caspase-3 positive cells per 40x field, 3 = 15–19 caspase-3 positive cells per 40x field, 4 = 20 caspase-3 positive cells per 40x field. Because the amount of apoptosis in the control animals varied by region, a degeneration score relative to the amount of background apoptosis was determined for each region of interest by subtracting the average degeneration score for each region of interest in the control conditions from the degeneration score of each experimental animal. This relative degeneration severity score for each region of interest was then analyzed by a repeated measure ANOVA.

Stereology and Dose-Response Analysis

Stereology was used to determine an estimate of the total number of cells undergoing apoptosis at different doses of DMSO. For this purpose a series of sections were chosen in a systematic random fashion from each brain (not including the brainstem and cerebellum) and processed for activated caspase-3 immunohistochemistry. The counter was blinded to litter, gender and treatment. The inter-section interval, counting frame size, and distance between counting frames were adjusted so that a reasonable number (approximately 200) of degenerating cells was sampled. The optical fractionator method was used to provide an unbiased estimate of the total number of apoptotic cells. Stereologic counting and estimates were done with the aid of StereoInvestigator version 7.5 (MicroBrightField, Inc, Colchester, VT). Guard volume was set to 5.0 μm . A one-way ANOVA with subsequent post-hoc comparisons were used to judge the significance of the observed effect. Regression analysis was conducted with the sigmoid equation model of Prism (Graphpad Software Inc., San Diego, CA).

In vitro hippocampal cultures

Dissociated mass cultures of P0–2 rat hippocampus were prepared as described previously (Mennerick et al., 1995). Briefly, hippocampal neurons were dissociated by papain and mechanical dispersion onto collagen-coated 35 mm plastic culture dishes. Culture growth medium was Eagle's medium (Invitrogen, Gaithersburg, MD) supplemented with heat-inactivated horse serum (5%), fetal calf serum (5%), 17 mM glucose, 400 μM glutamine, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin. Cultures were plated at 2000 cells/ mm^2 and contained both astrocytes and neurons. Cells were treated on DIV 3 with cytosine arabinoside (10 μM) to arrest glial division and at DIV 4 with the appropriate concentration of DMSO, added directly to the culture medium. In some dishes, a depolarizing concentration of KCl (30 mM) was simultaneously co-added to the dish. Cells were evaluated at DIV 10 with phase-contrast microscopy (20 $\times$ objective), and surviving neurons were counted in 10–20 randomly chosen fields as previously described (Moulder et al., 2002). Cell survival was expressed as cell counts in the experimental condition divided by control cell counts in sibling cultures, minus 1. This yielded a fractional change relative to control; negative numbers represent cell loss relative to control, and positive numbers represent better survival than control.

RESULTS

Initial Descriptive Study

Caspase-3 is the major effector caspase in the CNS and the immunohistochemical detection of cleaved (*i.e.* activated) caspase-3 immunoreactivity has been shown to be a sensitive indicator of apoptosis (Kuan et al., 2000). Therefore, to screen for apoptotic cell death, postnatal day (P) 7 C57BL/6 mouse pups ($n=78$) were injected with 10 ml/kg DMSO or saline, perfused at various post-injection time points, and brains were examined for activated caspase-3 immunoreactivity. In general animals tended to tolerate exposure to DMSO well with no gross decrement in the amount of wakefulness. After an hour or two the animals appeared to be able to nurse. In saline-treated animals occasional activated caspase-3 positive neurons were present, indicating that some neurons at this age undergo apoptosis naturally (Fig. 1A–D). In contrast, DMSO-treated animals had a dramatic elevation in the number of activated caspase-3 positive neurons and the degenerative process appeared to be relatively rapid. Two hours after DMSO exposure, some cells began to show detectable caspase-3 staining (Fig. 1E–H). Damage at this early time point was restricted mainly to the caudate/putamen (CPu) and some cortical regions (*e.g.* retrosplenial cortex and anterior cingulate cortex). At later time points (*i.e.* 4 and 8 hours) the staining was more robust in these early affected regions (Fig. 1I–P). In addition, the damage became more widespread at these two successive time points with degeneration becoming detectable throughout large regions of the brain. In the cortex, layer 2 neurons tended to become involved before layer 4 neurons. By 8 hours after injection, activated caspase-3

positive profiles were present in most cortical regions. Specific regions of the thalamus (*e.g.* lateral-dorsal), subiculum, lateral geniculate, and internal granule cell layer of the cerebellum were also severely affected. Greatest amounts of damage were in the cortex and CPu (Fig. 2). At 12 hours, while overall activated caspase-3 staining was increased (Fig. 1Q–T), closer detailed examination of the tissue revealed that a substantial amount of the staining was not localized to the cell body. At 24 hours after DMSO exposure, there was still a large amount of staining not localized to cell bodies but there was a further reduction in the number of positively stained cells (Fig. 1U–X). In addition, prominent activated caspases-3 staining was noted in white matter tracts. De Olmos silver and Nissl staining at 24 hours revealed the presence of fragmented pieces of cellular debris and apoptotic bodies, respectively (Fig. 3A–B), indicating that the degeneration was at an advanced stage. We next sought to confirm whether DMSO produced degeneration via an apoptotic mechanism using electron microscopy, the gold standard for detecting apoptosis histologically. Electron microscopy revealed that degenerating cells displayed ultrastructural hallmarks of apoptosis (Ishimaru et al., 1999; Kerr et al., 1972) -- loss of nuclear membranes, chromatin balls and condensation of the cytoplasm (Fig. 3C–E).

Dose Finding Study

To examine the dose response nature of DMSO-induced apoptosis, P7 mice ($n=70$) were exposed to one of several DMSO doses (0, 0.3, 1, 1.25, 3, and 10 ml/kg) and sacrificed 8 hours after injection because damage at this time point was maximal and cell bodies were still intact. The damage produced by DMSO is dose-dependent ($F[5, 57]=55.45$, $p<0.0001$; Fig. 4). However, at doses above 10 ml/kg, DMSO was highly toxic and substantial animal death occurred, making it impossible to develop a complete dose-response curve or reliably determine an ED_{50} . The lowest dose tested, 0.3 ml/kg, resulted in approximately 110,000 apoptotic neurons, an amount of damage that was more than 150% greater ($p=0.0017$) than the observed background rate of 70,000 seen in the control animals. At 10 ml/kg, it was estimated that over 900,000 neurons were undergoing apoptosis.

Age-Dependency Study

To explore whether animals of different ages are similarly vulnerable to the apoptotic effect of DMSO, we exposed infant and juvenile animals (P0, P3, P7, P10, P14, P17, P21, P30; $n=139$) to 10 ml/kg DMSO or saline and sacrificed the animals 8 hours later. The location and severity of apoptotic degeneration induced by DMSO varied with age (Table 1). At P0, the majority of degenerating cells were concentrated in the periventricular region, the anterior cingulate and retrosplenial cortex bordering the cingulum and the hippocampal commissure. Injured cells tended to be round with minimal processes suggesting that the cells were immature and still in the process of differentiating. Because these regions contain the migratory streams, it is likely that the cells are maturing neurons that are in transit to their final location in the brain. Degenerating profiles of more mature neurons were seen to a minor degree in certain thalamic nuclei, the amygdala, the superior and inferior colliculi, and the hypothalamus. At P3 degenerating profiles were still present not only in the same regions seen at P0, but also in new regions – CPu, cerebellum, and several additional thalamic nuclei and cortical regions. Also in the anterior cingulate and retrosplenial cortex, the apoptotic cells were mature and located at a distance from the cingulum. At P7, while the extent and overall severity of damage was greater than that seen at P3, the pattern of damage was different. Minimal damage was seen in those regions that were primarily affected at P0. Instead damage was greatest in those regions that had minor damage at P0 or that first began to show damage at P3. Additional areas involved included the subiculum, dentate gyrus, occipital cortex and the internal granule cell layer of the cerebellum. At P10, the pattern of degeneration, for the most part, was similar to that of P7 but was less severe, indicating that the greatest amount of overall damage occurred at P7. Degenerating profiles were also seen in the white matter regions of the cerebellum and CPu as well as in the internal capsule. By P14, the total burden of damage continued to lessen in the

gray matter with the greatest amount of damage being localized to the nucleus accumbens, dentate gyrus, lateral geniculate and the granule cell layers of the cerebellum. In contrast, damage in the white matter was more widespread than at P10. At P17, damage in the white matter continued to increase. In the gray matter, damage had lessened and was present mainly in some cortical regions and in the caudal aspects of the dentate gyrus. By P30, damage had decreased further and was restricted mainly to some cortical regions and white matter.

Hippocampal Culture Study

Previously we found that agents that produce apoptosis *in vivo* also cause apoptosis *in vitro* (Moulder et al., 2002). To determine if a similar effect is seen with DMSO, the solvent was added to the media of cultured hippocampal neurons at day *in vitro* (DIV) 4 to produce a final concentration of either 0.5% or 1%. Compared to untreated cultures, DMSO-treated cultures exhibited increased neurodegeneration ($p < 0.05$ for both 0.5% and 1% DMSO versus untreated cultures, $n = 4$ experiments, Fig. 5). Although 1% DMSO showed a trend toward greater neuronal loss than 0.5% DMSO, this increase was not statistically significant ($n = 4$ experiments).

Our previous results suggest that potassium-induced depolarization protects against apoptosis induced by ethanol and NMDA antagonists (Moulder et al., 2002). Depolarization, thought to act through moderate rises in intracellular calcium concentration, is also known to protect many neuron types from apoptotic loss *in vitro* (Mennerick and Zorumski, 2000). Similarly, the neurodegeneration induced by 1% DMSO was overcome by a depolarizing increase in KCl concentration added to the culture media ($p < 0.05$ $n = 4$ experiments, Fig. 5). Taken together the results suggest that DMSO damage shares features with the apoptosis elicited by ethanol and NMDA receptor antagonists and that the damage in cell culture is likely to be apoptotic. As noted previously (Moulder et al., 2002; Shute et al., 2005), KCl was also protective against background attritional cell loss in the cultures (Fig. 5).

PCP Comparison Study

Previously studies have shown that several NMDA antagonists as well as ethanol, which has NMDA antagonist properties, produce apoptosis in the developing rodent brain (Dribben et al., 2008; Ikonomidou et al., 2000; Ikonomidou et al., 1999; Scallet et al., 2004; Wang et al., 2001; Wang and Johnson, 2007). DMSO has also been reported to antagonize NMDA receptors at a concentration that we found produces apoptosis in *in vitro* hippocampal neurons (Lu and Mattson, 2001). This suggests that DMSO could be producing apoptosis via blockade of NMDA receptors. To explore this possibility, we exposed P7 mice ($n=15$) to the NMDA antagonist, phencyclidine (PCP, 50 mg/kg), and compared the pattern of apoptotic degeneration 8 hours after exposure to that seen with DMSO. Consistent with previous studies, PCP produced widespread apoptosis. All regions that were undergoing apoptosis in the PCP-treated animals also were undergoing apoptosis in the DMSO-treated animals but the damage was more severe in the DMSO group. In addition, a couple of regions of the brain (*e.g.* hypothalamus and Islands of Calleja), developed apoptosis in the DMSO-treated animals. However, these regions were not damaged in the PCP-treated animals (Fig. 6).

DISCUSSION

In this study, we report that DMSO produces widespread, dose-dependent neurodegeneration in the developing CNS as ascertained by activated caspase-3 immunohistochemistry. Electron microscopy confirmed that degenerating neurons displayed histological hallmarks of apoptosis. While DMSO has been noted to produce apoptosis in lymphoid tissue *in vitro* (Chateau et al., 1996; Trubiani et al., 1996) as well as *in vivo* (Aita et al., 2005), this is the first report, to our knowledge, that DMSO produces apoptosis in the CNS. At the time of peak

sensitivity – P7 –we could detect significant apoptosis at the lowest dose tested, 0.3 ml/kg, and at the maximal dose tested approximately 900,000 neurons were undergoing apoptosis. Given that damage in some regions (*e.g.* CPu) is no longer detectable 8 hours after injection, this number is likely to be an underestimate. The dose of 0.3 ml/kg is approximately 10 fold lower than the toxic dose reported for lymphoid tissue (Aita et al., 2005). In an *in vitro* preparation, we found that concentrations as low as 0.5% could produce apoptosis.

While the observed damage was widespread, not all regions of the brain were sensitive and sensitivity depended on the age of the animal at the time of exposure. In addition, even within a specific region, the population of cells vulnerable to the damage changed over time. For example, in the retrosplenial cortex, damaged cells at P0 were small cells without processes that were close to the cingulum. At P7, the apoptotic cells appeared to be mature pyramidal neurons in specific layers. Consistent with apoptosis induced by other agents (*e.g.* ethanol, NMDA receptor antagonists, GABAergic agents) we found that damage in the CPu and hippocampus occurred earlier than damage in the thalamus and cortex, which tended to predominate beginning around P7. The overlap in regions damaged by DMSO and PCP, our *in vitro* findings, and the fact that DMSO has been reported to inhibit NMDA receptors (Lu and Mattson, 2001), suggest that some of the observed damage could result from blockade of NMDA receptors. However, two additional observations suggest that NMDA receptor blockade might not completely account for the observed damage. First, the period during which the developing CNS is vulnerable to DMSO's pro-apoptotic action is longer than with PCP. With other NMDA antagonists the window of vulnerability ends around P14 (Dribben et al., 2008; Ikonomidou et al., 2000; Ikonomidou et al., 1999). DMSO continued to produce substantial damage even at the oldest age tested, P30, an age equivalent to late childhood in humans. At the older ages, damage in white matter tracts tended to predominate. Further work is needed to determine whether even older animals are sensitive to DMSO's pro-apoptotic effects. It is assumed that the apoptotic cells in the white matter are of glial lineage but further work will be needed to confirm this assumption. Second, it appeared that DMSO produced apoptosis in a couple of brain regions that are not injured by PCP, despite administering PCP at near lethal doses. Thus, we were unable to test fully the extent of damage produced by PCP because of tolerability, and we cannot rule out the possibility that an even higher degree of NMDA receptor blockade could produce damage in these other regions. These two differences suggest that while DMSO might produce some of the damage via inhibition of NMDA receptors, DMSO could be inducing damage through different and, as yet, unknown mechanisms. The effect of DMSO on dissociated cells suggests that toxicity is likely a direct effect on neurons rather than an indirect peripheral action with secondary neuronal effects. Furthermore, the *in vitro* data suggest that intact neuronal circuitry is not required for the neurodegenerative effects of DMSO. Studies utilizing oligodendroglial or astrocytic cultures would be important to pursue in determining the mechanism underlying the observed apoptosis in older animals.

DMSO has had a long and checkered past. It has been routinely available as a commercial solvent since the 1950s and was initially studied for clinical use in the 1960s, but testing was halted after concerns about chronic exposure inducing eye lens abnormalities surfaced (Noel et al., 1975; Rubin, 1983; Rubin and Mattis, 1966). Testing in humans resumed a year later but with restrictions. DMSO was finally approved by the United States Food and Drug Administration (FDA) for clinical use in 1978 as a treatment for interstitial cystitis where it is introduced into the bladder for 20–30 minutes and then removed. It has been proposed as a treatment for a wide array of ailments – scleroderma, amyloidosis, contact dermatitis, rheumatoid arthritis, allergic eczema, pain syndromes, elevated intracranial pressure, soft tissue damage, and schizophrenia -- and has been proposed as a bacteriostatic, fungistatic, diuretic, cholinesterase inhibitor and free radical scavenger (Muir, 1996; Santos et al., 2003). However, consistent results from well controlled trials documenting efficacy are

lacking. As of 2008, there are three studies listed in ClinicalTrials.gov in which DMSO is being directly investigated for therapeutic use. One involves intravesical application for painful bladder syndromes (NCT00583219). The other two involve transdermal applications for basal cell carcinoma (NCT00218829) and melanoma (NCT00118313). Whether DMSO will become an accepted treatment for any of these indications is unknown. While DMSO is not approved by the FDA for marketing as a systemic treatment for any illness, DMSO remains a commercial solvent that can be easily obtained from a wide variety of sources over the Internet, allowing people to treat themselves at will.

In spite of the fact that DMSO has not shown consistent efficacy for any clinical condition that requires systemic application, humans are exposed systemically to this agent routinely in clinical settings where it is used as a cryoprotectant in the processing of tissue for stem cell transplantation. In bone marrow transplantation, DMSO is added to the bone marrow prior to freezing. The concentration of DMSO in the cryoprotectant is between 3.25 and 20%, with the most typical concentration being 10% (Windrum et al., 2005). After thawing, the cells along with the DMSO are injected into the patient. This procedure is used routinely in children who receive bone marrow transplantation as a part of their treatment for neuroblastoma and Ewing's sarcoma. Used as a cryoprotectant, the average dose of DMSO has been found to be 0.63 ml/kg (mean; [Davis et al., 1990]), 1.8 ml/kg (median; [Curcoy et al., 2002]) and 0.9 ml/kg (mode; [Windrum et al., 2005]). Given that we observed apoptosis even at the lowest dose tested, 0.3 ml/kg, exposure to DMSO during transplantation could be producing similar damage in children. While there is growing recognition that DMSO may have side effects (Hubel, 2001; Stroncek et al., 1991), minimal attention is being paid to potential neurotoxic effects. For example, the current on-going NIH supported study evaluating DMSO toxicity in stem cell transplants (NCT00631787) is not evaluating any neurotoxic effects (<http://www.clinicaltrials.gov/ct2/show/NCT00631787?term=dms0&rank=7>). Moreover while case reports have associated DMSO with neurological side effects, including seizures, transient global amnesia, and cerebral infarction in adults (Hoyt et al., 2000; Junior et al., 2008; Mueller et al., 2007; Windrum and Morris, 2003), there is only one adverse case report in a child. In that case, a 16 year old developed reversible leukoencephalopathy after DMSO exposure (Higman et al., 2000). Despite the lack of data, it remains unclear whether DMSO is safe in children. Unfortunately, there are also no data in rodents that examine the behavioral and cognitive effects of neonatal DMSO exposure. Two published studies (Authier et al., 2002; Fossum et al., 1985) report behavioral effects of DMSO but these studies were done in adult rats when it is unclear whether DMSO-induced apoptosis occurs. Thus, it will be important to conduct studies to determine whether DMSO, when given to young animals at doses known to cause apoptosis, produces long-lasting changes in cognition or behavior.

Until data with DMSO become available, it is perhaps useful to extrapolate from experiments assessing cognitive and behavioral outcomes of other agents that produce developmental apoptosis. Ethanol is the most extensively studied of all the agents that have been found to produce this type of damage in the *in vivo* rodent. Ethanol-induced apoptosis is considered to be the likely mechanism to account for some of the CNS sequelae that are associated with Fetal Alcohol Syndrome (Olney et al., 2002a). In this case, a single, several hour exposure to ethanol in P7 mice results in substantial apoptosis and produces detectable impairments in spatial learning and memory when the animals are tested as juveniles (Wozniak et al., 2004). The damage observed with DMSO is at least as severe as that seen with ethanol, suggesting that DMSO-induced apoptosis might also produce significant learning and memory deficits. Given that a brief exposure to ethanol produces apoptosis in non-human primate brain (Farber et al., 2005), the infant human brain might be at risk of developing apoptosis with similar exposure. Studies to further clarify the risk to human infants are needed.

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Figure 1. DMSO-Induced Caspase-3 Immunoreactivity at Different Post-Survival Intervals

Activated caspase-3 immunohistochemistry from P7 mouse pups treated with either saline (A–D) or DMSO (E–X) and sacrificed at various time points (2, 4, 8, 12, & 24 hours) after exposure to DMSO. Left column contains low-powered photomicrographs, all of which are taken from approximately the same rostro-caudal level. The three columns on the right show contain high-powered photomicrographs from caudate/putamen (CPu), lateral dorsal thalamus (LD Thal) and retrosplenial cortex (RSC). The approximate location from which each of the high-powered photomicrographs is taken is indicated by an asterisk in panel A. Subtle increases in activated caspase-3 activity could be seen by 2 hours. This increase is difficult to detect on high-powered images (black arrows = activated caspase-3 positive cells) but it is evident when comparing

panels E and A. Peak damage occurred at 8 hours in most areas of the brain. However, some areas (*e.g.* CPu) showed peak staining at 4 hours. While the overall amount of activated caspase-3 appeared to be greater at 12 and 24 hours compared to 8 hours when comparing low-powered photomicrographs (Q and U vs. M), high-powered photomicrographs (R–X) showed that while some of the staining at 12 and 24 hours was localized to intact cell bodies (white asterisks), a larger amount of the staining appeared to come from debris (white arrows) and a general increase in background staining (white arrowheads). Prominent staining of white matter tracks was noted at 24 hours (U; black arrowheads). The Levels function of Photoshop® was used to lighten the amount of dark staining in images T and X in order to allow the cell bodies to be more easily seen.

Figure 2. Activated Caspase-3 Immunoreactivity Varies by Region

P7 animals were treated with either DMSO 10 ml/kg (n=9) or saline (n=10) and sacrificed 8 hours later. Relative degeneration severity scores were determined for several different regions as indicated in Methods. Zero indicates similar degeneration relative to the same region in control animals. Regions have been ranked ordered by decreasing severity scores. The severity of activated caspase-3 immunoreactivity varied between different regions ($F[11,88]=10.62$, $p<0.0001$). The difference was mainly due to damage in the cortex (Ctx) and caudate/putamen (CPu) being greater than the other sampled regions of the brain (Newman-Keuls). Error bars = SEM.

Figure 3. DMSO-Induced Apoptotic Degeneration

Nissl (A) and De Olmos silver (B) stained sections of the CPu taken from a P7 animal exposed to DMSO (10 ml/kg) 24 hours previously. A) Numerous darkly labeled spheroid balls (black arrowheads), or apoptotic bodies, are present in the Nissl section. B) At 24 hours silver stain demonstrates that the degeneration is in an advanced stage. Cell bodies (white arrowheads) are amorphous indicating that cell membranes are no longer intact. Axons and dendrites are no longer discernible. Electron microscopic micrographs of the ultrastructure of degenerating cells after exposure to DMSO (C–F), demonstrate that the cells have the classical hallmarks of apoptotic degeneration. C) A neuron early in the apoptotic degenerative process. At this stage, the mitochondria (m) are slightly swollen. The nuclear membrane (black arrows) is still intact and separates the cytoplasm, which is slightly condensed, from the nucleoplasm. In the nucleus, chromatin has begun to clump into a chromatin ball (cb). D) Photomicrograph of a neuron in the later stages of apoptotic degeneration. The nuclear membrane is no longer intact, allowing the nuclear and cytoplasmic compartments to mix. The neuron has become more condensed and the chromatin ball is spherical and is in the center of the cell. The cell membrane (black arrows) remains relatively intact and the cell appears to be surrounded by processes from a phagocytic cell. E) Image of a neuron in a very late stage of apoptotic degeneration. The chromatin body has become marginalized and the material inside the cell is becoming degraded. While the dense degraded cell can still be distinguished from the surrounding neuropil, the

cellular membrane is beginning to be compromised by processes from the surrounding phagocytic cell, which have begun to encroach into the degenerating cell (white arrow).

Figure 4. Dose-Dependency of DMSO-Induced Apoptosis

Animals were treated with one of several doses of DMSO and sacrificed 8 hours later. Total number of caspase-3 positive neurons was estimated with stereology. The number of degenerating cells increased with increasing doses of DMSO ($p<0.0001$) with R^2 of 0.82. The lowest dose tested produced a significantly greater amount of apoptosis than was present in the control animals ($p=0.0017$). Numbers in parentheses indicate number of animals studied at each dose. Error bars (SEM) for most points are too small to be seen.

Figure 5. DMSO-Induced Neuronal Loss in Hippocampal Culture

DMSO at either 0.5% or 1.0% produces a substantial decrease in the number of cultured hippocampal neurons ($p < 0.05$). The loss of neurons is prevented by the addition of KCl. 0 (solid line) represents control counts ($p < 0.05$). -1.0 (dotted line) represents 100% loss. +1.0 would represent doubling of cell numbers over control. Note that KCl increases neuronal survival over baseline (Moulder et al., 2002; Shute et al., 2005) and largely prevents the additional cell loss induced by DMSO. Error bars = SEM.

Figure 6. Comparison of PCP-Induced and DMSO-Induced Apoptosis

Activated caspases-3 immunohistochemistry of sections from three different rostrocaudal levels taken from animals treated with either DMSO (10 ml/kg; left hemisections) or PCP (50 mg/kg; right hemisections) and sacrificed 8 hours later. DMSO appeared to produce more apoptosis than PCP even though the doses for both agents were approaching toxic levels. The pattern of damage was similar in most regions of the brain, consistent with the agents producing apoptosis via a similar mechanism. There were some exceptions to this rule, however. A large number of neurons were degenerating in the Islands of Calleja (ICj; circled region) with DMSO. In contrast in the PCP-treated animals this region was not affected. Similarly, several regions of the hypothalamus (anterior [AntHypoTh] and medial [MedHypoTh] areas are illustrated here) were severely affected. In contrast, no damage above control levels was detected in the PCP-treated animals.

Table 1

DMSO-Induced Apoptosis By Region at Different Ages

Region	Age at Exposure					
	P0	P3	P7	P10	P14	P17
Medial Habenula	X					
Lateral Septum						
AV Thalamus						
Reticular Thalamus		X				
Hippocampal commissure	X					
Neuroepithelium						
Indusium Griesium						
Amygdala						
Ventral Medial Hypothalamus	X	X	X			
Superior Colliculus						
Inferior Colliculus	X	X	X	X		
Nucleus Accumbens	X	X	X	X	X	
Posterior Thalamic Nuclei	X	X	X	X	X	X
Anterior Cingulate Cortex						
Retrosplenial Cortex	X	X	X	X	X	X
Dorsal Medial Hypothalamus						
Zona Incerta		X				
Olfactory Tubercle						
CA1/CA3		X	X			
Entorhinal Cortex						

Region	Age at Exposure						
	P0	P3	P7	P10	P14	P17	P30
Piriform Cortex							
Lateral Dorsal Thalamus							
Lateral & Medial Geniculate		X	X	X	X		
Caudate/Putamen		X	X	X	X	X	
Posterior Hypothalamus							
Parietal Cortex							X
Frontal Cortex							
Temporal Cortex		X	X	X	X	X	
Orbital Cortex							
Anterior Hypothalamus			X	X			
Occipital Cortex							
Subiculum			X	X	X		
Cerebellum							
Dentate Gyrus			X	X	X	X	
Insula			X	X	X	X	X
Taenia Tecta				X			
Medial Orbital Cortex							
Ventral Anterior Thalamus							
Ventral Medial Thalamus				X	X		X
Endopenducular Nucleus							
Olfactory Nucleus							