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IN VIVO AND *IN VITRO* KETAMINE EXPOSURE EXHIBITS A DOSE-DEPENDENT INDUCTION OF ACTIVITY-DEPENDENT NEUROPROTECTIVE PROTEIN IN RAT NEURONS

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Abstract—Anesthetic doses of ketamine induce apoptosis, as well as gene expression of activity-dependent neuroprotective protein (ADNP), a putative homeodomain transcription factor in rat pups (P7). This study investigated if ketamine induced ADNP protein in a dose-dependent manner *in vitro* and *in vivo* using primary cultures of cortical neurons and neonatal pups (P7). *In vivo* immunohistochemistry demonstrated a sub-anesthetic dose of ketamine increased ADNP in the somatosensory cortex (SCC) which was previously identified to be damaged by repeated exposure to anesthetic doses of ketamine. Administration of low-dose ketamine prior to full sedation prevented caspase-3 activation in the hippocampus and SCC. Primary cultures of cortical neurons treated with ketamine (10 μM–10 mM) at 3 days-*in vitro* (3 DIV) displayed a concentration-dependent decrease in expanded growth cones. Furthermore, neuronal production and localization of ADNP varied as a function of both ketamine concentration and length of exposure. Taken together, these data support the model that ADNP induction may be partially responsible for the efficacy of a low-dose ketamine pre-treatment in preventing ketamine-induced neuronal cell death.

Key words: activity dependent protective protein, ketamine, cortical neurons, growth cones.

INTRODUCTION

Ketamine is on the World Health Organization's List of Essential Medicines for Children up to 12 years of age (2013). Ketamine is a dissociative anesthetic, and its potential for help or harm in children is controversial (Bhutta, 2007; Bhutta et al., 2007; Davidson, 2011). Ketamine is a non-competitive N-methyl-D-aspartate (NMDA) antagonist that disrupts calcium homeostasis in neurons (Sinner et al., 2005; Slikker et al., 2007). Since calcium signaling has an important role in cytoskeleton stability (Lankford and Letourneau, 1989) as well as neuronal viability (Turner et al., 2007; Ringler et al., 2008), ketamine-induced calcium deregulation may lead to alterations in axonal outgrowth and induction of apoptotic cell death (Wang et al., 2005; Turner et al., 2012; Bai et al., 2013; Dong and Anand, 2013).

This study investigated the dual role of ketamine, as both a neurotoxin in some animal models (Wang et al., 2005; Slikker et al., 2007; Turner et al., 2012; Bai et al., 2013), and as a neuroprotectant in other animal models (Anand et al., 2007; Rovnaghi et al., 2008; Turner et al., 2012). Depending on the dosage, ketamine has been demonstrated to be protective as well as destructive to certain brain regions. The putative transcription factor activity-dependent neuroprotective protein (ADNP) gene expression increased 1.5-fold in the somatosensory cortex (SCC) of post-natal day 7 (P7) rats following a sedative dose of ketamine or MK801 (Turner et al., 2012). Thus we sought to inquire about the potential neuroprotective role of ADNP in this model system. If *ADNP* is indeed upregulated during ketamine sedation, it may be that sub-anesthetic doses of ketamine increase cellular levels of ADNP. Additionally, xenon, another NMDA antagonist (Liu et al., 2010) with demonstrated neuroprotective properties (Wilhelm et al., 2002), induced *ADNP* gene expression in neonatal rats (Cattano et al., 2008). Thus, increased *ADNP* led us to consider to what extent ADNP, a protein of increasing importance as a potential therapeutic target, may be regulated by ketamine.

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Abbreviations: AASS, antibiotic antimycotic solution stabilized; AC3, activated caspase 3; ADNF, activity-dependent neurotrophic factor; ADNP, activity-dependent neuroprotective protein; ASD, autism spectrum disorder; BCA, bicinchoninic acid; DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast; DIV, days-*in vitro*; EB, end-binding; FC, fasciola cinereum; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MK801, dizocilpine; NAP, amino acid sequence NAPVSIPQ; NMDA, N-methyl-D-aspartate; P7, post-natal day 7; PBS, phosphate-buffered saline; PBST, PBS with 0.025%Tween 20; PFA, paraformaldehyde; PSD, post-synaptic density protein 95; SSC, somatosensory cortex.

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Developmentally *ADNP* mRNA is expressed at E7 and peaks between E9.5–14 just prior to neural tube closure in mice (Pinhasov et al., 2003). When ADNP is not present in transgenic mice, the neural tube failed to close, an embryonic lethal phenotype (Pinhasov et al., 2003). Even mice with only half the concentration of ADNP demonstrate increased neurodegeneration with increased age (Vulih-Shultzman et al., 2007). ADNP's mechanism of action may be a regulator of gene transcription during early development (Pinhasov et al., 2003) or a regulator of splicing and transcription through its potential binding with heterochromatin protein (HP) 1α (Mandel et al., 2007) and Brahma (Brm), a component of the SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodeling complex (Schirer et al., 2014). Additional evidence for ADNP's function correlated a human mutation in exon 5 of ADNP with autism and increased expression of *ADNP-1* and *ADNP-2* mRNA (Helsmoortel et al., 2014). ADNP is currently the most prevalent genetic mutation predicted in autism spectrum disorder (ASD) cases (Helsmoortel et al., 2014). Thus understanding ADNP protein production, localization and molecular regulation is critical to determining its role in nervous system development and disease.

Much of the research related to the physiological effects of ADNP has been conducted by investigating the mediating effects of NAPVSIPQ (NAP). NAP is an 8-amino acid peptide contained within ADNP (Bassan et al., 1999) that has acquired interest for clinical use because it has a similar structure to the neuroprotective 9-amino acid peptide derived from activity-dependent neurotrophic factor (ADNF-9) (Bassan et al., 1999). Results from studies conducted on ADNF-9 demonstrate that peptide fragments of parent proteins may also have protective effects (Brenneman and Gozes, 1996; Brenneman et al., 1998). In fact NAP replacement minimized the detrimental effects of ADNP reduction via the prevention of tau hyperphosphorylation (Vulih-Shultzman et al., 2007), decreased p53 (Gozes and Divinski, 2004), and the enhancement of neural tube closure during embryonic development (Bassan et al., 1999). Various independent neuroprotective and positive effects of NAP, include inhibition of the caspase-3 activation (Leker et al., 2002), prevention of the release cytochrome c, an early inducer of apoptosis (Zemlyak et al., 2009b), and assistance in microtubule assembly (Divinski et al., 2006) as well as neurite extension (Lagreze et al., 2005; Smith-Swintosky et al., 2005). A potential mechanism for the neural protective capability of NAP is a direct interaction with microtubule-stabilizing proteins in the axon (Gozes and Divinski, 2007).

Interestingly, *in vivo* NAP blocked ketamine-induced injury (20 mg/kg) in the somatosensory cortex (layers IV–V; $SCC_{IV–V}$) as measured by activated caspase 3 (AC3)-positive cell increase (Turner et al., 2012). Similarly, low-doses of ketamine (5 mg/kg) administered prior to high-doses of ketamine (20 mg/kg) demonstrated a significant reduction in apoptotic cells of the $SCC_{IV–V}$ and the retrosplenial cortex (Turner et al., 2012). We suspected that in the latter instance low-dose ketamine acts to produce protective effects analogous to those observed following NAP injection. We hypothesized that because ketamine increases the expression of *ADNP*, and because both ADNP and its peptide derivative, NAP, are implicated in cell survival, a low-dose ketamine pretreatment would induce endogenous ADNP and thus prevent injury caused by a subsequent high dose of ketamine.

EXPERIMENTAL PROCEDURES

All chemicals used in the research were from Sigma–Aldrich, MO unless stated otherwise, and all media used for cell culture were from Invitrogen.

Treatment of ketamine and brain isolation

Administration of ketamine and brain tissue preparation methods were adopted and modified from Turner et al. (2012). For preconditioning with ketamine, P6 rat pups were injected subcutaneously with a low dose of ketamine (5 mg/kg) or saline for control 24 h before a higher, anesthetic dose of ketamine. On P7, rat pups were injected with 50 μL ketamine (20 mg/kg) four times over 3 h (Turner et al., 2012). Eight hours after the first P7 injection, all animals were anesthetized with 2% isoflurane and then perfused with 0.9% heparin/phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in PBS. Brains were cut into 90-μm-thick coronal sections with a microtome (Leica SM 2000R, Germany) and stored in cryoprotectants (30% sucrose, 0.1 M pH 7.4 phosphate buffer, and 30% ethylene glycol) at −20 °C until further processed. All experimental groups had three animals and each experiment was replicated three times.

Immunohistochemistry

Brain sections were processed for antigen retrieval prior to incubation with the primary antibody using sodium citrate (10 mM, pH 6.0) at 92–95 °C for 4 min and cooled down immediately, then incubated for 5 min on ice. After washing with PBS, sections were incubated with either Rb α-AC-3 (1:1000; R&D, Minneapolis, MN, USA) or Rb α-ADNP (1:1000, Bethyl laboratory, TX, USA, Catalog #IHC-00118) followed by a biotinylated goat α-Rb (1:200; Vector Labs, Burlingame, CA, USA). Detection with antibodies was determined using an Elite avidin–biotin complex (ABC) kit with VIP chromagen solutions (both from Vector Labs, CA), and sections were mounted, air-dried, dehydrated in ascending ethanol concentrations, and coverslipped with Permount (Sigma–Aldrich, MO, USA). Controls performed without primary antibody did not demonstrate specific staining with primary antibody. For ADNP detection, antigen retrieval was omitted. All assays were performed with a negative control by omitting the primary antibody.

Stereological quantification

All histological sections were counted using non-biased quantitative investigation stereology as shown by a previous study (Gutierrez et al., 2010). Quantification of AC-3 positive neurons and ADNP-positive neurons were