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REGION-SPECIFIC ROLE FOR GLUN2B-CONTAINING NMDA RECEPTORS IN INJURY TO PURKINJE CELLS AND CA1 NEURONS FOLLOWING GLOBAL CEREBRAL ISCHEMIA

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Abstract—Motor deficits are present in cardiac arrest survivors and injury to cerebellar Purkinje cells (PCs) likely contribute to impairments in motor coordination and post-hypoxic myoclonus. N-Methyl-D-aspartic acid (NMDA) receptor-mediated excitotoxicity is a well-established mechanism of cell death in several brain regions, but the role of NMDA receptors in PC injury remains understudied. Emerging data in cortical and hippocampal neurons indicate that the GluN2A-containing NMDA receptors signal to improve cell survival and GluN2B-containing receptors contribute to neuronal injury. This study compared neuronal injury in the hippocampal CA1 region to that in PCs and investigated the role of NMDA receptors in PC injury in our mouse model of cardiac arrest and cardiopulmonary resuscitation (CA/CPR). Analysis of cell density demonstrated a 24% loss of PCs within 24 h after 8 min CA/CPR and injury stabilized to 33% by 7 days. The subunit promiscuous NMDA receptor antagonist MK-801 protected both CA1 neurons and PCs from ischemic injury following CA/CPR, demonstrating a role for NMDA receptor activation in injury to both brain regions. In contrast, the GluN2B antagonist, Co 101244, had no effect on PC loss while protecting against injury in the CA1 region. These data indicate that ischemic injury to cerebellar PCs progresses via different cell death mechanisms compared to hippocampal CA1 neurons.

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Abbreviations: ACSF, artificial cerebral spinal fluid; ANOVA, analysis of variance; CA/CPR, cardiac arrest and cardiopulmonary resuscitation; CREB, cAMP response element-binding; EGTA, ethylene glycol tetraacetic acid; H&E, hematoxylin and eosin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LCM, laser capture microdissection; MK-801, dizocilpine; NMDA, N-methyl-D-aspartic acid; PCs, Purkinje cells; PFA, paraformaldehyde; RT-PCR, reverse transcription polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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INTRODUCTION

Cardiac arrest is a leading cause of death and disability with approximately 600,000 cardiac arrests occurring annually in the United States (Go et al., 2013). Neurological injury resulting from cardiac arrest is the most common cause of mortality and morbidity among those who are successfully resuscitated (Laver et al., 2004; Lim et al., 2004). Transient global ischemia resulting from cardiac arrest and cardiopulmonary resuscitation (CA/CPR) results in death of selectively vulnerable populations of neurons (Ng et al., 1989; Horn and Schlote, 1992; Kofler et al., 2004). Rodent models of cerebral ischemia have been used extensively to examine injury mechanisms and neuroprotective therapies in the cortex, striatum and hippocampus (Merchenthaler et al., 2003; Traystman, 2003; Kofler et al., 2004; Noppens et al., 2008). However, fewer studies have focused on the cerebellum following global ischemia, despite data indicating that cerebellar Purkinje cells are highly vulnerable to cerebral ischemia (Ng et al., 1989; Horn and Schlote, 1992; Welsh et al., 2002; Paine et al., 2012). Movement disorders are often observed in cardiac arrest survivors and are attributed primarily to striatal damage, but it is likely that cerebellar injury also contributes to motor deficits in post-cardiac arrest patients (Venkatesan and Frucht, 2006; Lu-Emerson and Khot, 2010). Purkinje neurons are the sole output of the cerebellar cortex and play a critical role in motor coordination, motor learning, and aspects of cognition (Gilbert and Thach, 1977; Llinás and Welsh, 1993). They receive and integrate excitatory inputs from sensory, vestibular and motor areas to allow for temporally and spatially precise movement (Thach, 1998; Bastian, 2006). There is evidence of Purkinje cell loss in post-mortem examinations of cardiac arrest victims and in rodent models of global cerebral ischemia however, the mechanism of injury to these cells remains understudied (Horn and Schlote, 1992; Welsh et al., 2002; Lim et al., 2004; Kelley et al., 2011).

Global ischemia results in neuronal death in the CA1 region of the hippocampus with a delayed onset of 2–3 days (Pulsinelli et al., 1982; Kofler et al., 2004). Mechanisms contributing to death of these neurons include excitotoxicity, oxidative stress and activation of

apoptotic pathways (Martin et al., 1998; Saito et al., 2005; Hertz, 2008). Excitotoxicity resulting from over activation of glutamate receptors, particularly N-methyl-D-aspartic acid (NMDA) receptors, is an early trigger for calcium dysregulation and cell death and has been the target of neuroprotective strategies (Newell et al., 1995; Arundine and Tymianski, 2004; Dhawan et al., 2011). Glutamate receptor antagonists can minimize injury of hippocampal CA1 and striatal neurons *in vitro* and *in vivo* mouse models of cerebral ischemia (Gill et al., 1987; Foster et al., 1988; Collins et al., 1989; Rothman and Olney, 1995), however, less data exist clarifying which receptors are responsible for glutamate toxicity in Purkinje cells following cerebral ischemia. There is evidence that glutamate excitotoxicity contributes to Purkinje cell death as AMPA receptor antagonists can protect against Purkinje cell loss following global cerebral ischemia (Balchen and Diemer, 1992; Brasko et al., 1995) and knockdown of glial glutamate transporter expression increases Purkinje cell loss following experimental cardiac arrest (Yamashita et al., 2006). Interestingly, recent studies using memantine, a non-competitive NMDA receptor antagonist have yielded conflicting results as to whether blocking NMDA activity prevents Purkinje cell injury following cardiac arrest (Tai and Truong, 2007, 2010, 2013). It is important to note that until recently, it was generally accepted that Purkinje cells lacked NMDA receptor expression, however recent work demonstrates a role for NMDA receptors in normal synaptic transmission and plasticity in Purkinje cells (Piochon et al., 2007, 2010; Renzi et al., 2007; He et al., 2013) which leads us to hypothesize that NMDA receptor activation may be a major contributor to Purkinje cell death. The current study aims to increase our understanding of the role of NMDA receptors in Purkinje cell injury following global cerebral ischemia.

A subunit specific role for NMDA receptor contribution to excitotoxic neuronal death has been proposed based on *in vitro* and *in vivo* animal studies. Synaptic GluN2A-containing receptors can promote cell survival following an excitotoxic insult, while extrasynaptic GluN2B-containing receptors promote cell death (Lynch and Guttmann, 2002; Liu et al., 2007; Choo et al., 2012; Sanz-Clemente et al., 2013). While there is evidence of opposing roles in ischemic injury of GluN2A and GluN2B containing NMDA receptors in cortical and hippocampal neurons *in vitro* and *in vivo*, NMDA receptor subunit-dependent mechanisms have not been examined in the cerebellum.

In this study we compared NMDA receptor subunit contributions to ischemic injury in CA1 neurons and Purkinje cells and revealed regional differences in injury mechanisms following experimental CA/CPR.

EXPERIMENTAL PROCEDURES

For all experiments, 8–12-wk male (20–25 g) C57Bl/6 mice (Charles River laboratories, Portage, MI, USA) were used. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Colorado, School of Medicine and were performed according to the guidelines from the National Institutes of Health. Mice were individually housed and allowed free access to food and water.

Cardiac arrest model

Cardiac arrest and cardiopulmonary resuscitation were performed as previously described (Kofler et al., 2004; Neigh et al., 2004) with slight modifications to head and body temperature. Mice were anesthetized initially with 3% isoflurane and maintained with 1.5–2% isoflurane in O_2 enriched air via facemask (Isotec 5, Ohmeda, Reno, NV, USA). Isoflurane exposure was consistent between groups (duration and dose) to ensure any preconditioning effects were uniform and not contributing to differences observed between treatment groups (Zheng and Zuo, 2003). Head and body temperatures were monitored using tympanic and rectal probes connected to separate automatic temperature controllers (Doric Instruments, San Diego, CA, USA) to maintain 37 ± 0.2 °C during surgery using a head coil, heating pad and a heating lamp. For drug administration, a PE-10 catheter was inserted into the right jugular vein and flushed with heparinized 0.9% saline solution. Needle electrodes were placed subcutaneously on the chest for EKG monitoring (Medical Data Electronics, Arleta, CA, USA) and mice were endotracheally intubated and connected to a mini ventilator (Harvard Apparatus, Holliston, MA, USA). To induce cardiac arrest, 50 µl of 0.5 M KCl was administered via the jugular vein. Cardiac arrest was confirmed by a flat line EKG and the endotracheal tube was then disconnected from the ventilator. During CA, a tympanic temperature of 37.5 °C and a rectal temperature of 35 °C were maintained. Following 8 min of cardiac arrest, resuscitation was performed by mechanical ventilation (190 breaths/min), injections of epinephrine (200 µl, 16 µg/ml in 0.9% saline; maximal dose 1 ml) and simultaneous chest compressions (300 compressions/min). As soon as there was a return of spontaneous circulation (ROSC), compressions were terminated. When spontaneous breathing reached a rate of 30 breaths/min, mechanical ventilation was stopped and the endotracheal tube removed. Temperature probes and catheters were then removed, and the skin wounds were closed. Drug administration (saline, MK-801 and Co 101244) was performed intravenously 30 min after resuscitation. Mice were returned to their home cages that were placed on a heated water blanket (35 °C) for recovery and received soft chow and free access to water. At various times after resuscitation (3 h–30 days), mice were transcardially perfused with 4% paraformaldehyde (PFA) and post-fixed in PFA at 4 °C overnight. Cerebellums and hippocampi were paraffin embedded and 6-µm sections, at 100-µm intervals, were cut and collected and stained for histology and immunohistochemistry.

Histology

The hippocampus and cerebellum were analyzed by hematoxylin and eosin (H&E) and Fluoro-Jade B at 3 h, 1 and 3 days after CA/CPR. For pharmacological studies, neuronal injury in the hippocampus and cerebellum was analyzed 7 days after CA/CPR.