Research Proposal: Neuroprotective Effects of R-Type Voltage Sensitive Calcium Channel Antagonists in Traumatic Brain Injury

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Writer's comment: I have worked in Dr. Robert Berman's neurosurgery laboratory at the UCD Center for Neuroscience for two years. While studying neuronal injury mechanisms in traumatic brain injury, I have learned that communication skills are an essential part of a researcher's life. Scientific knowledge does not exist until researchers communicate their knowledge—not only among themselves but also with the rest of the world. In English 104E, Dr. Pamela Demory encouraged the class to understand the needs of audiences with various backgrounds in order to effectively communicate scientific knowledge. In writing a grant proposal, I learned to aim for a mixed audience that includes people with very different expertise. I also enjoyed designing practical experiments based on my research experience and literature reviews. Lastly, I would like to thank Dr. Demory for her assistance and for giving me this wonderful opportunityto submit my paper to *Prized Writing.*

—Izumi Toyoda

Instructor's comment: This assignment asks students to write a proposal for original research to the National Science Foundation, using the NSF guidelines. The writer must appeal to the goals and values of the NSF, as well as appealing to the multiple audiences that would evaluate such a proposal: the non-specialist administrators, the general specialists in the field, and the experts in the particular area of research. Izumi's proposal is stellar. One of the most impressive things about her writing is her ability to present complex research clearly—in the Introduction and Conclusion she explains her research for a non-specialist audience and still maintains a high level of specificity; in her Background and Methodology sections, she writes for more specialized audiences, including a greater level of technical detail, and still manages to maintain her lucid style. Equally impressive is the proposal's persuasiveness: her experimental design is well-thought-out, and her rationale is clear and forceful.

—Pamela Demory, English Department

Introduction

Traumatic brain injury (TBI) is one of the leading causes of death and disability in the United States. Approximately 1.5 to 2 million people in the United States suffer from TBI each year, chiefly as a result of vehicle crashes, falls, firearms, and sport injuries. Unlike age related neurodegenerative disorders such as Parkinson's, Alzheimer's, and Huntington's disease, TBI could happen to anyone at any place and any age. Although severe TBI frequently results in death, people who survive from moderate to mild TBI often develop life-long impairments of physical, cognitive, and psychological functions.

For many decades, researchers have been trying to understand the pathophysiology of TBI. Disruption of calcium homeostasis has been proposed to play a key role in brain injury mechanisms since calcium mediates critical cellular processes. In the central nervous system, voltage-sensitive calcium channels (VSCCs) are the major sources of calcium entry into both pre- and postsynaptic terminals. Six subtypes of VSCCs (P/Q, N, L, R, T) have been sequenced and characterized, and their respective antagonists have been synthesized (Catterall, 2000). Although the neuroprotective properties of P/Q-, N-, and L-type VSCC antagonists have been studied extensively, very few studies have examined the neuroprotective effects of SNX-482, an R-type VSCC antagonist, which was discovered recently (Newcomb et al., 1998). Therefore, the purpose of this proposal is to examine the role of R-type VSCCs in the pathology of neurodegeneration and the neuroprotective effects of SNX-482 following TBI. The experiments are designed to examine a hypothesis that blockade of R-type VSCCs following TBI can reduce the degree of neuronal injury and improve behavioral outcomes.

Background

A. Neurotoxicity of Altered Calcium Homeostasis and Excess Glutamate

The pathophysiology of TBI is separated into two stages: primary and secondary. Primary injury refers to the immediate physical damage caused by the mechanical insult to the head, which deforms or shears brain tissue. Primary injury initiates secondary injury mechanisms, which involve biochemical cascades leading to delayed injury to cells. Understanding of these biochemical cascades will lead to better understanding of injury mechanisms, which may aid in the development of sensible therapeutics for head trauma.

One of the principle theories that describe the possible biochemical cascades is the "calcium hypothesis," which asserts that neuronal

calcium overload leads to subsequent neurodegeneration (Siesjo, 1981). Normally, the intracellular free calcium concentration is kept very low and is tightly regulated by many mechanisms such as membrane receptors, ion channels, ion exchangers, calcium storage organelles, and calcium binding proteins. However, TBI causes an excessive entry of calcium into the cell, which disrupts the calcium homeostasis. Toxicity of altered calcium homeostasis was first suggested by McLean et al. (1965), who observed calcium accumulation in damaged hepatocytes. Later, Schanne et al. (1979) concluded that calcium influx was the "final common pathway of cell death" and hypothesized that calcium influx is required for cell injury and death. However, precise mechanisms of this calcium neurotoxicity are still unknown.

An increase in intracellular calcium concentration triggers neurotransmitter release and activates numbers of cellular enzymes. Neurotoxicity of excess glutamate, a major excitatory neurotransmitter in the central nervous system, had been proposed even before Siesjo proposed the "calcium hypothesis." For example, Olney (1969) confirmed the neurotoxicity of glutamate by showing that glutamate produces brain lesions when injected into immature animals. Then, Choi (1987) suggested the connection among calcium, glutamate, and cell death; he demonstrated that exposure to excess glutamate results in calcium-mediated delayed cell death. Today, it is widely accepted that glutamate toxicity is largely calcium-dependent.

B. R-type VSCC Antagonists and Neuronal Injury

Researchers have been interested in voltage-sensitive calcium channels (VSCCs) because of their ability to regulate calcium-influx into excitable cells and to initiate neurotransmitter release. Although the role of VSCCs in TBI is still unclear, discovery of VSCCs in the central nervous system has encouraged the examination of therapeutic potentials of VSCC antagonists in brain injury. Many VSCC antagonists have been synthesized to examine their neuroprotective effects and the role of VSCCs in TBI. While P/Q-type VSCC antagonists are known to be toxic, several L-, N-, and T-type VSCC antagonists have shown positive neuroprotective effects and are currently in clinical trials for human TBI (Newcomb et al., 2000).

The R-type VSCC current was identified as the current that "remained" in the cerebellar granule cells after blockade with L-, N-, and P/Q-type VSCC antagonists. Unlike antagonists for P/Q-, N-, L-, and T-type VSCCs, a selective R-type VSCC antagonist was not identified

until recently. Consequently, functional roles for R-type VSCCs are poorly understood although several studies have suggested their roles in glutamate-release and neuronal transmission (Wu et al., 1998; Gasparini et al., 2001). Because calcium influx through R-type VSCCs is closely associated with increased glutamate-release, their antagonists may prevent neurodegeneration by reducing glutamate-release following TBI. However, Toriyama et al. (2002) have recently suggested that activation of R-type VSCCs following ischemia is not neurodegenerative but neuroprotective instead. Ischemia resulted in higher intracellular calcium concentration in mutant mice lacking R-type VSCCs than in wild-type controls. Although mechanisms in which R-type VSCCs reduced intracellular calcium concentration in ischemic mice are unknown, R-type VSCCs are likely candidates for the future therapeutic targets (Toriyama et al., 2002). The proposed study of neuroprotective effects of R-type VSCC antagonists will elucidate the role of R-type VSCCs in the pathophysiology of TBI and further investigate the therapeutic potentials of SNX-482.

Methodology

A. Lateral Fluid Percussion in Rat TBI models

Lateral fluid percussion (LFP) will be used to produce brain injury in rats. The rat fluid percussion model was originally characterized by Dixon et al. (1987) and later modified by McIntosh et al. (1989). It produces reliable and reproducible brain injury in rodents, which is similar to that observed in human head trauma. Before the injury, rats will be anesthetized with 2% isoflurane in a 2:1 N2O/O2 carrier gas mixture, intubated and mechanically ventilated with a rodent volume ventilator. Then, craniotomy will be performed over the right parietal cortex to expose an intact dura, which will be connected to the fluid percussion device. All procedures will follow the American Association for the Accreditation of Laboratory Animal Care guideline for the humane treatment of laboratory animals.

B. Injection of the R-type VSCC Antagonist, SNX-482

SNX-482 is the first known selective R-type VSCC antagonist, which has been isolated from the venom of the African tarantula, *Hysterocrates gigas* (Newcomb et al., 2000), and it effectively blocks the activation of R-type VSCCs (Bourinet et al., 2001). Following brain injury, animals in the drug-treated group will receive stereotaxic injec-

tions of a solution containing SNX-482 while those in the control group will receive artificial cerebral spinal fluid (ACSF)-vehicle. To ensure that the magnitude of injury is not affected by either the surgical procedure or the drug itself, the sham-uninjured group will be compared to the TBI group. The sham-uninjured group will undergo the identical anesthesia and surgery as the TBI animals but will not receive the fluid percussion to the brain, and they will receive either a solution containing SNX-482 or ACSF-vehicle.

C. Histological Analysis of Neuronal Degeneration

The degree of acute neuronal degeneration in the hippocampus will be histologically analyzed since the hippocampus is a part of the limbic system, which affects learning and memory. At 24 hours post-TBI, both SNX-482-treated and ACSF-vehicle treated animals will be sacrificed and perfused transcardially with phosphate buffer and 4% paraformaldehyde to fix the brain. Brains will be removed, sectioned, mounted onto slides, and stained with Fluoro-Jade B. Fluoro-Jade B is an anionic fluorochrome, which stains degenerating neurons. Its high affinity for degenerating neurons and low background staining make Fluoro-Jade B a better staining agent than its predecessor, Fluoro-Jade (Schmued and Hopkins, 2000). Fluoro-Jade B positive neurons will be observed by fluorescence microscopy and quantified by manual counting.

D. Behavioral Analysis following TBI

Neurobehavioral outcome following TBI and blockade of R-type VSCCs will be assessed using motor and cognitive performance tests. Berman et al. (2000) used the following behavioral tests and successfully assessed the neuroprotective effects of SNX-111, N-type VSCC antagonist.

1. Inclined Plane Test. An inclined plane will be used to test muscle strength of animals. Animals will be placed onto the inclined platform with different angles, and the maximum angle on which they can cling to the platform for 10 seconds will be recorded. Brain injured animals with motor deficits have difficulty remaining on the inclined plane at steep angles.Therefore, the test will assess the severity of neurodegeneration, which results in deficits in muscle strength following TBI.

2. Beam Walk Test. Beam walk will be used to examine vestibular function, fine motor coordination, and ambulatory functions before

and after injury. Animals must cross a long and narrow beam and enter an escape box at the end of the beam in order to escape from the light and noise at the starting point, and time required to cross the beam is recorded. Long latencies to cross the beam will indicate deficits of fine motor coordination.

3. Radial Arm Maze. The radial arm maze, which was developed by Olton et al. (1977), will be used to test spatial working memory. The maze consists of 8 arms radiating away from a central start box, and a small piece of food is placed at the end of each arm as a reward. The amount of time required by the animals to enter all arms to eat the reward and the numbers of errors will be recorded. Errors are defined as repetition of an arm, failure to eat reward, and failure to enter an arm. The radial arm maze tests spatial working memory since the animals must remember the spatial location of the arms that they have visited already. The animals are trained in the radial arm maze before injury, and then they will be tested again following TBI to assess their memory for previously acquired knowledge.

4. Morris Water Maze. The Morris water maze will be used to test the acquisition of spatial reference learning (Morris, 1984). It consists of a large circular pool of water with a submerged platform. The animals must learn to find the submerged platform in order to escape from a forced swimming task, and the latency of escape and swim speed will be recorded. The animals will be tested in the Morris water maze following TBI without pre-training. Therefore, the Morris water maze will test their ability to acquire new spatial memory following TBI.

Overall Significance of the Proposed Research

The proposed research is designed to further the understanding of roles of R-type VSCCs in neuronal degeneration following TBI. Histological analysis of neuronal degeneration will assess the neuroprotective effects of R-type VSCC antagonists at cellular level while behavioral analysis will assess those at neurobehavioral level. Moreover, the degree of neurodegeneration observed in the histological analysis can be directly associated with the neurobehavioral outcomes using the proposed behavior tests. Although several VSCC antagonists have been used in clinical trials for neurotrauma, none of these antagonists have shown significant therapeutic effectiveness so far. Because of the increasing number of TBI-related deaths and disability in the United

States, the search for better therapeutics for brain injury must be continued. Better understandings of VSCC functions and examination of their antagonists aid in the development of sensible therapeutic strategies for TBI and thus promote the national health, prosperity, and welfare.

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