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Treatment with afobazole at delayed time points following ischemic stroke improves long-term functional and histological outcomes

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ABSTRACT

There is currently a significant lack of therapeutic options for acute ischemic stroke, and no drug has been approved for treating patients at delayed time points (≥6 h post-stroke). Afobazole, an anxiolytic currently used clinically in Russia, has been shown to reduce neuronal and glial cell injury *in vitro* following ischemia. Experiments using the permanent middle cerebral artery occlusion (MCAO) rat model were carried out to determine if afobazole can reduce ischemic stroke damage *in vivo* and expand the therapeutic window for stroke treatment. Post-stroke (24 h) application of afobazole (0.3–3 mg/kg) significantly decreased infarct volume at 96 h post-surgery, as determined by Fluoro-Jade and NeuN staining of brain sections. Moreover, afobazole helped preserve both the levels and normal histological distribution of myelin basic protein, indicating a reduction in white matter injury. A time-dependence study showed that either pre-treatment or treatment started 6 to 48 h post-stroke with the drug yields improved outcomes at 96 h. The decrease in infarct volume produced by afobazole was blocked by the application of either a σ-1 (BD 1063, 30 mg/kg) or a σ-2 (SM-21, 1 mg/kg) antagonist, indicating that both receptor subtypes are involved in the effects of afobazole. Treatment with afobazole starting at 24 h post-stroke resulted in enhanced survival one month following surgery. Behavioral testing of animals 28–32 days post-surgery using the elevated body swing and forelimb grip-strength tests revealed that treatment with afobazole starting 24 h post-stroke significantly reduces behavioral deficits caused by ischemic stroke. The increase in survival and improved functional outcomes are accompanied by a reduction in infarct volume, as determined by thionin staining of brain sections. Taken together, our data support the use of afobazole as a post-stroke pharmacological agent to expand the current therapeutic window.

Introduction

Stroke is the second leading cause of death world-wide according to the World Health Organization, accounting for nearly 11% of all deaths, which translates to over 6,000,000 individuals per year (WHO, 2011). In addition, stroke is a leading cause of long-term disability, and places an economic burden of over $38 billion per year in the United States alone. The American Heart Association predicts that the number of deaths due to ischemic stroke will likely double between 2002 and 2032. The poor outcomes associated with stroke are in part due to the lack of therapeutic alternatives for patients, with only a single drug, recombinant tissue plasminogen activator (rtPA), available for acute stroke treatment. Due to various contraindications and the narrow therapeutic window in which the drug may be used (<4.5 h post stroke), less than 5% of stroke patients are eligible for rtPA treatment (Allen et al., 2012). Therefore, it is of great interest to identify novel drug targets and effective pharmacological agents for the treatment of this disease.

Afobazole (5-ethoxy-2-[2-(morpholino)-ethylthio]benzimidazole) is an anxiolytic drug that was first approved for clinical use in Russia in 2005. Studies have suggested that afobazole may be neuroprotective in various pathophysiological models. For example, *in vitro* studies have shown that afobazole can decrease neuronal death caused by oxidative stress and glutamate excitotoxicity (Zenina et al., 2005). *In vivo* studies have shown that afobazole can increase survival rates and decrease neurological deficits in rats suffering from a post-traumatic hematoma (Galaeva et al., 2005). Similarly, afobazole was shown to be neuroprotective in rats after bilateral local photothrombosis of vessels in the prefrontal cortex and after global transient ischemia (Baykova et al., 2011; Seredenin et al., 2008).

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These prior studies motivated work in our laboratory to explore the use of afobazole for limiting injury following focal ischemic injury to the brain. Recent studies in our laboratory showed that afobazole affects cellular responses to ischemia *via* the stimulation of σ receptors (Cuevas et al., 2011a, 2011b). Activation of σ-1 receptors by afobazole blocks intracellular calcium overload produced by ischemia and acidosis and reduces neuronal death after *in vitro* ischemia (Cuevas et al., 2011a). This activation of σ-1 receptors results in the block of multiple ion channels that are functionally upregulated following ischemia and acidosis, including acid-sensing ion channel 1a (ASIC1a) (Cuevas et al., 2011a). In microglia, activation of both σ-1 and σ-2 receptors by afobazole reduces membrane ruffling and migration of these cells in response to ATP (Cuevas et al., 2011b). Moreover, afobazole prevents microglial cell death following ischemia, even when the drug was applied after the ischemic insult (Cuevas et al., 2011b). Therefore, *in vitro* studies suggest that afobazole, acting on σ receptors, can affect multiple processes involved in the demise of brain tissue following an ischemic insult.

Both subtypes of σ receptors, σ-1 and σ-2, are widely distributed in the mammalian central nervous system. *In vivo* studies have demonstrated the potential of σ receptors as targets for stroke therapy. Our laboratory showed that activation of σ receptors *in vivo* decreases neuronal injury in a rat model of large-vessel ischemic stroke, even when these receptors are activated 24 h after onset of ischemia (Ajmo et al., 2006). These original findings have now been supported by work in other laboratories using other σ receptor ligands (Li et al., 2009; Ruscher et al., 2011). The *in vivo* findings have supported *in vitro* observations which suggest that σ receptor activation decreases stroke injury by providing neuroprotection and by suppressing the inflammatory response which accompanies brain ischemia (Ajmo et al., 2006). There is some controversy, however, as to the long-term benefits of σ receptor activation following stroke. One study showed that the pan-selective σ receptor agonist DTG fails to decrease infarct volume and improve behavioral outcomes one month post-stroke in rats (Leonardo et al., 2010). In contrast, the σ-1 selective agonist, SA4503, was found to improve functional recovery four weeks after MCAO in rats, but did not affect stroke volume (Ruscher et al., 2011).

Data presented here demonstrate that the pan-selective σ receptor agonist, afobazole, decreases infarct volume at both early (96 h post-stroke) and late (1 month post-stroke) stages following ischemia in the MCAO rat model. Afobazole is effective even when application of the drug is initiated at delayed time points (≥6 h post-stroke). Afobazole decreases neuronal and glial cell death and reduces the neuroinflammatory response. Moreover, the decrease in cell injury results in enhanced survival of the animals and improved behavioral outcomes even 1 month post-stroke.

Materials and methods

MCAO surgery

Adult male Sprague–Dawley rats weighing 325–375 g (Harlan, Indianapolis, IN) were housed in a climate-controlled facility and cared for according to the NIH Guide for the Care and Use of Laboratory Animals and protocols approved by the Institutional Animal Care and Use Committee at the University of Florida. Water and laboratory rat food were available *ad libitum*. Rats were anesthetized (5%–2% isofluorane in 95–98% O_2 at 1 L/min) and given pre-op injections of ketofen (11.5 mg/kg), atropine (0.1 mg/kg) and enrofloxacin (10 mg/kg). A 500 μm fiber optic laser Doppler probe (Moor Instruments, Devon England) was inserted into a hole drilled through the skull 1 mm posterior and 4 mm lateral to Bregma to monitor cerebral blood flow using MoorLAB software. After making an incision along the throat, the common carotid (CCA), external carotid (ECA) and internal carotid (ICA) Arteries were isolated and clamped. A 4 cm monofilament (250 μm diameter) was inserted into the ligated ECA and advanced distally into the ICA to the origin of the middle cerebral artery (MCA) as determined by a reduction in cerebral blood flow. A successful occlusion of the MCA required at least a 40% reduction in the Laser Doppler signal. The monofilament and ECA were ligated and the throat incision closed. The Doppler probe was removed, the hole in the skull was filled with bone wax and the scalp incision closed. Sham surgeries were identical to MCAO surgeries except for the insertion of the monofilament. Following surgery, animals received daily subcutaneous injections of ketofen for 3 days. All animals in the long-term study were also injected daily with 3 mL of saline subcutaneously for 3 days to help with survival.

Treatment protocols

Intraperitoneal injections of afobazole or vehicle (saline) were given 3 times daily in all studies. For dose–response studies, injections of the indicated doses were initiated 24 h post-surgery and continued for 3 days, until animals were sacrificed at 96 h post-stroke. Animals receiving sham surgery in the dose-dependent study received 30 mg/kg afobazole injections. In time-dependent studies, 1 mg/kg afobazole was injected starting at the indicated post-stroke time point and continued until 96 h post-stroke. Animals in the pre-treatment group, in both the time-dependent and long-term behavior studies, were injected with afobazole for 7 days prior to surgery and then starting again 3 h post-surgery. For the σ receptor antagonist studies, animals received the antagonist (BD 1063, 10 mg/kg; SM-21 1 mg/kg) alone or in combination with afobazole (3 mg/kg) starting at 24 h post-stroke. Animals in the sham group of the long-term study received 3 mg/kg afobazole 3× per day. Animals were randomly assigned to treatment groups, except in the behavioral studies where animals were segregated such that test groups had similar distributions of pre-op behavioral performance ratings. Upon completion of the study periods, animals were injected with ketamine (75 mg/kg) and xylazine (7.5 mg/kg), perfused with 0.9% PBS followed with 4% paraformaldehyde, and brains were removed. Brains were equilibrated in 30% sucrose, and sectioned frozen on a cryostat at 30 μm thickness in the coronal plane. Six sections were collected from each brain at equal intervals, from the frontal striatum to the dorsal hippocampus (coordinates: Bregma 1.7 to Bregma −3.3), as described previously (Ajmo et al., 2006). Sections were cryopreserved at −20 °C for subsequent immunohistochemistry and histology.

Fluoro-Jade staining

Frozen brain sections were dried at 45 °C for 50 min, and stained with Fluoro-Jade (Millipore, Billerica, MA) according to manufacturer's recommendations. Fluoro-Jade positive signal, indicative of neurodegeneration, was quantified by image analysis with Image J (NIH). The Fluoro-Jade positive area for each brain section was normalized to the total area of the contralateral hemisphere to compensate for brain edema. The areas for the six Bregma point sections collected from each brain were combined to generate an infarct volume for the brain. In all experiments involving image analysis, the individual(s) taking the photomicrographs and conducting the analysis was blinded as to the treatment group of the animal being studied.

NeuN and myelin basic protein immunohistochemistry

Six brain sections collected as described above were stained with NeuN antibody (Millipore, Billerica, MA; 1:3000). NeuN labeling was visualized using a fluorescent labeled secondary antibody (Goat anti-mouse, Alexa Fluor 488, Molecular Probes, Eugene, OR; 1:300). Two sections, one representing the core (Bregma −0.3) and a second the outer segments (Bregma 1.7) of the infarct, were analyzed with Image J to determine the total number of NeuN-positive neurons. For each section, three images were captured in the ipsilateral striatum at 100×, and the total number of neurons per visual field was determined.