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Neuroprotection and reduced gliosis by atomoxetine pretreatment in a gerbil model of transient cerebral ischemia

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ABSTRACT

Atomoxetine (ATX) is a non-stimulant selective norepinephrine reuptake inhibitor that is widely used for the treatment of attention-deficit/hyperactivity disorder (ADHD). In this study, we firstly examined neuroprotective effects of pre- or post-treatment with 15 and 30 mg/kg ATX against ischemic damage in the gerbil hippocampal cornus ammonis 1 (CA1) region subjected to 5 min of transient cerebral ischemia using cresyl violet staining, neuronal nuclei immunohistochemistry and Fluoro-J B histofluorescence staining. We found that only pre-treatment with 30 mg/kg ATX protected CA1 pyramidal neurons from ischemic insult. In addition, pre-treatment with 30 mg/kg ATX, which had neuroprotective effect against ischemic damage, distinctly attenuated the activation of astrocytes and microglia in the ischemic CA1 region compared with the vehicle-treated ischemia group by glial fibrillary acidic protein (for astrocytes) and ionized calcium-binding adapter molecule 1 (for microglia) immunohistochemistry. In brief, our present results indicate that ATX has neuroprotective effect against transient cerebral ischemic insult and that the neuroprotective effect of ATX may be closely associated with attenuated glial activation.

1. Introduction

Transient cerebral ischemia evokes neuronal death in some specific brain regions, such as the stratum, neocortex and hippocampus [1,2]. Especially, the cornus ammonis 1 (CA1) region of the hippocampus is well known to be selectively vulnerable to transient ischemic insult, and neuronal death in this region is called "delayed neuronal death (DND)", because the neuronal death does not occur immediately but takes place several days after initial ischemic insult [3,4]. The underlying mechanisms for the DND are complicated and multiple; some mechanisms related with the DND have been suggested to include glutamate-induced neurotoxicity [5], glia-mediated neuroinflammation [6] and oxidative stress by excessive free radical generation [7]. However, the accurate mechanisms responsible for the DND are still not fully elucidated.

It has been reported that neuropsychiatric medications have some beneficial effects through multiple molecular mechanisms beyond their original effectiveness in brain diseases including cerebral ischemic insults [8–10]. Atomoxetine (ATX, Strattera), a selective norepinephrine reuptake inhibitor, is a non-stimulant medication that is most commonly used to treat attention-deficit/hyperactivity disorder (ADHD) in both children and adult patients [11,12]. Beyond the main role of ATX as a norepinephrine reuptake inhibitor, it shows ability to decrease glutamate transmission by antagonizing N-methyl-D-aspartate (NMDA) receptor [13], upregulates the expression of brain-derived neurotrophic factor (BDNF) and BDNF-mediated signaling [14], and attenuates oxidative damage [15]. In addition, ATX provides beneficial effects in experimental animal models of vascular dementia and traumatic brain injury [16,17]. However, so far there are no reports about effects of ATX on cerebral ischemic insults. In this study, therefore, we examined effects of ATX against transient cerebral ischemia in the hippocampal CA1 region

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induced by 5 min of transient cerebral ischemia in gerbils, which are very effectively used as a useful animal model for transient cerebral ischemia research [9,18].

2. Materials and methods

2.1. Experimental animals

We obtained male Mongolian gerbils (*Meriones unguiculatus*) from the Experimental Animal Center, Kangwon National University, Chuncheon, South Korea and used them at 6 month (body weight, 75–80 g) of age. The animals were maintained in pathogen-free conditions under temperature (23 °C) and humidity (60%) with a 12-h light/12-h dark cycle and were provided with free access to food and water. All the experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Kangwon University and adhered to guidelines that are in compliance with the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th Ed., 2011). All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

2.2. Treatment with atomoxetine

To elucidate protective effects of ATX (Eli Lilly, Indianapolis, IN) against ischemic damage after ischemia–reperfusion, the animals were divided respectively as follows ($n = 7$ at each point in time in each group): 1) sham-group, which received sham operation; 2) and 3) pre- and post-vehicle-ischemia-group, which was treated with saline before and after ischemia–reperfusion, respectively; 4), 5), 6) and 7) 15 and 30 mg/kg pre- and post-ATX-ischemia-group, which was treated with 15 and 30 mg/kg ATX before and after ischemia–reperfusion, respectively. ATX was dissolved in saline and intraperitoneally administrated once daily for 3 days before or after the ischemic surgery: The last pretreatment and the first posttreatment, respectively, were at 30 min before and after the surgery.

In preliminary study, we examined the protective effect of pre- or post-treatments with 15, 30 and 50 mg/kg ATX, and significant effects were shown in animals pre-treated with 30 and 50 mg/kg; there was no difference in the protective effects between 30 and 50 mg/kg. Therefore, we chose 15 and 30 mg/kg ATX in the present study.

2.3. Induction of transient cerebral ischemia

The gerbil model of transient cerebral ischemia was established according to a previously published method by us [19]. In brief, the animals were anesthetized with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. Both common carotid arteries were occluded using non-traumatic aneurysm clips (Yasargil FE 723K, Aesculap, Tuttlingen, Germany). The complete interruption of blood flow was confirmed by observing the central artery in retinae using an ophthalmoscope (HEINE K180®, Heine Optotechnik, Herrsching, Germany). After 5 min of occlusion, the restoration of blood flow (reperfusion) was observed directly using the ophthalmoscope. Body (rectal) temperature was maintained under free-regulating or normothermic (37 ± 0.5 °C) conditions with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA) and a thermometric blanket before, during and after the surgery until the animals completely recovered from anesthesia. Sham-operated animals were subjected to the same surgical procedures except that the common carotid arteries were not occluded.

2.4. Tissue processing for histology

As we described previously [19], briefly, the gerbils were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter, frozen tissues were serially sectioned on a cryostat (Leica, Germany) into 30-μm coronal sections.

2.5. CV staining

To investigate the morphological and neuronal changes in the hippocampus of each group, cresyl violet (CV) staining was carried out as we described previously [19]. Cresyl violet acetate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved at 1.0% (w/v) in distilled water, and glacial acetic acid was added to this solution. The sections were stained and mounted with Canada balsam (Kanto, Tokyo, Japan).

2.6. F-J B histofluorescence staining

To examine neuronal damage/death in the CA1 region, Fluoro-Jade B (F-J B, a high affinity fluorescent marker for the localization of neuronal degeneration) histofluorescence staining was established according to a previously published method by us [20]. In brief, the sections were first immersed in a solution containing 1% sodium hydroxide in 80% ethanol, and followed in 70% ethanol. They were then transferred to a solution of 0.06% potassium permanganate, and transferred to a 0.0004% F-J B (Histochem, Jefferson, AR) staining solution. After washing, the sections were placed on a slide warmer (approximately 50 °C), and then examined using an epifluorescent microscope (Carl Zeiss, Göttingen, Germany) with blue (450–490 nm) excitation light and a barrier filter.

2.7. Immunohistochemistry for NeuN, GFAP and Iba-1

In order to examine neuronal damage and glial activation in the hippocampus, immunohistochemistry was carried out according to a previously published method by us [21]. We used mouse anti-neuronal nuclei (NeuN, diluted 1:1000, Chemicon, USA) for neurons, mouse anti- glial fibrillary acidic protein (GFAP, diluted 1: 800, Chemicon, USA) for astrocytes and rabbit anti- ionized calcium-binding adapter molecule 1 (Iba-1, 1:800, Chemicon, USA) for microglia. In brief, the sections were incubated with each primary antibody, exposed to biotinylated goat anti-mouse or rabbit IgG and streptavidin peroxidase complex (Vector, USA), and visualized with 3,3′-diaminobenzidine in 0.1 M Tris HCl buffer.

2.8. Data analysis

Numbers of NeuN-immunoreactive and F-J B positive cells were analyzed according to the previously published method by us [20]. In brief, digital images of the hippocampus were captured with an AxioM1 light microscope (Carl Zeiss, Germany) equipped with a digital camera (Axiocam, Carl Zeiss, Germany) connected to a PC monitor. The cells were counted in a 250 × 250 μm square applied approximately at the center of the hippocampal CA1 region using an image analyzing system (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ). The studied tissue sections were selected with 120-μm interval, and cell counts were obtained by averaging the total cell numbers of 8 sections taken from each animal per group.

In addition, as we previously described [21], densities of GFAP and Iba-1-immunoreactive structures were measured. Images of all GFAP- and Iba-1-immunoreactive structures were taken from three layers (strata oriens, pyramidale and radiatum) through an AxioM1 light microscope (Carl Zeiss, Germany) equipped with a digital camera (Axiocam, Carl Zeiss) connected to a PC monitor. Images were calibrated into an array of 512 × 512 pixels corresponding to a tissue area of 200 × 200 μm (40× primary magnification) including the stratum pyramidale. The densities of all GFAP- and Iba-1-immunoreactive structures were evaluated on the basis of an optical density (OD), which was