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Rufinamide, an antiepileptic drug, improves cognition and increases neurogenesis in the aged gerbil hippocampal dentate gyrus via increasing expressions of IGF-1, IGF-1R and *p*-CREB

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ABSTRACT

Rufinamide is a novel antiepileptic drug and commonly used in the treatment of Lennox-Gastaut syndrome. In the present study, we investigated effects of rufinamide on cognitive function using passive avoidance test and neurogenesis in the hippocampal dentate gyrus using Ki-67 (a marker for cell proliferation), doublecortin (DCX, a marker for neuroblast) and BrdU/NeuN (markers for newly generated mature neurons) immunohistochemistry in aged gerbils. Aged gerbils (24-month old) were treated with 1 mg/kg and 3 mg/kg rufinamide for 4 weeks. Treatment with 3 mg/kg rufinamide, not 1 mg/kg rufinamide, significantly improved cognitive function and increased neurogenesis, showing that proliferating cells (Ki-67-immunoreactive cells), differentiating neuroblasts (DCX-immunoreactive neuroblasts) and mature neurons (BrdU/NeuN-immunoreactive cells) in the aged dentate gyrus compared with those in the control group. When we examined its mechanisms, rufinamide significantly increased immunoreactivities of insulin-like growth factor-1 (IGF-1), its receptor (IGF-1R), and phosphorylated cAMP response element binding protein (*p*-CREB). However, rufinamide did not show any increase in immunoreactivities of brain-derived neurotrophic factor and its receptor. Therefore, our results indicate that rufinamide can improve cognitive function and increase neurogenesis in the hippocampus of the aged gerbil via increasing expressions of IGF-1, IGF-1R and *p*-CREB.

1. Introduction

Neurogenesis in the CNS is most active during pre-natal development, and the neurogenesis decreases substantially with age [1]. Similarly, lots of aged individuals suffer from cognitive impairment, such as memory decay [2]. The hippocampus is important for learning and memory, and hippocampal function and histological integrity deteriorate with age [3]. The subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) is a major part of the brain involved in adult neurogenesis, and the decline of the neurogenesis in the aged may result in neurodegenerative diseases [4]. It is well known that new neurons in the brain are progressively generated in the subventricular zone of the lateral ventricles and in the SGZ of the DG [5]. Neuronal stem cells proliferate, differentiate, and become integrated into neuronal

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Hippocampal neurogenesis is positively influenced by several growth factors, such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1) and vascular endothelial cell growth factor (VEGF) through the activation of transcription factor cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), which is a critical signal for neurogenesis and related to the formation of long-term learning and memory [6]. The phosphorylation of CREB appears to be very sensitive to the concentration of BDNF [7]. BDNF is involved in cognitive function and synaptic plasticity [8]. In particular, the upregulation of BDNF promotes cellular proliferation via BDNF receptor TrkB [9]. In addition, it has been reported that IGF-1 is involved in the regulation of neurogenesis and cognitive function via activating CREB by binding with IGF-1 receptor (IGF-1R) [10,11].

Rufinamide (RUF, 1-[(2,6-difluorophenyl)methyl]-1H-1,2,3-triazole-4 carboxamide) is a novel antiepileptic drug and commonly used in the treatment of Lennox-Gastaut syndrome [12]. Antiepileptic drugs, such as lacosamide [13] and levetiracetam [14], promote neuroblast differentiation and neurogenesis in the hippocampus. It has been reported that RUF improves cognitive and behavioral deficits in diabetic neuropathy [15]. To the best of our knowledge, however, studies regarding effects of RUF on neurogenesis in the hippocampal DG have not been reported yet.

In the present study, therefore, we investigated effects of RUF on neurogenesis of aged hippocampal DG using Ki-67 and doublecortin (DCX) and BrdU immunohistochemistry as well as cognitive function using passive avoidance in aged gerbils. In addition, we explored expressions of BDNF, TrkB, IGF-1 and IGF-1R, which display important roles in neurogenesis, in the DG after RUF administration in the gerbil which is a good model of aging [16,17].

2. Materials and methods

2.1. Experimental animals

We used aged male Mongolian gerbils (*Meriones unguiculatus*) at 24 months (B.W., 80–90 g) of age, which were obtained from the Experimental Animal Center, Kangwon National University, Chuncheon, Republic of Korea. The animals were housed in a conventional cage under adequate temperature (23 °C) and humidity (60%) control, 12-hour light/dark cycle, and free access to water and food according to the 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). The experimental protocol of this study was approved by the Institutional Animal Care and Use Committee (IACUC, approved no. KW-160802-1) at Kangwon National University.

2.2. Treatment with RUF and BrdU

Gerbils were divided into three groups (n = 7 in each group): 1) vehicle-group, which was treated with saline (0.9% w/v NaCl), 2) 1 mg/kg RUF-group and 3) 3 mg/kg RUF-group, which were treated with 1 mg/kg of RUF and 3 mg/kg of RUF, respectively, according to Kharatmal et al. [15]. RUF was dissolved in normal saline and administered intraperitoneally once daily for 4 weeks before sacrifice.

All the animals were received intraperitoneal injection of 50 mg/kg of BrdU (Sigma, St. Louis, MO) to label mitotic cells in the DG on day 8, 15, 22 and 27 during the experiment according to our published procedure [18,19]. During the treatment of RUF, abnormal behavior, dietary change, and weight changes are used to determine the health status of the animals. The animals were weighed twice a week during the experiment, and no differences in health between the vehicle- and RUF-groups were shown.

2.3. Passive avoidance test

To examine learning and memory, passive avoidance test was performed 30 min after vehicle or RUF administration for the last 3 days before sacrifice according to a method by Ahn et al. [20]. We used the Gemini Avoidance System (GEM 392, San Diego Instruments), which was consisted of two compartments (light and dark) with a grid floor, to perform this test. In short, trial task for the test was performed for 2 days. Each gerbil was allowed to adapt environments in A and B dark compartments for 1 min at which point the door was opened. In the next 1 min, light was turned on only in the A compartment and the gerbil explored in the A and B compartments. When the gerbil entered the B-compartment, the door was closed and given an inescapable footshock (0.5 mA for 5 s). In test task, 24 h later, the light was turned on when the gerbil was placed in the A compartment, and we kept the door open. We recorded latency time for passive avoidance between the beginning of the test and the entry into the dark compartment. The latency time to enter the B-compartment was recorded within 180 s.

2.4. Tissue processing for histology

The animals were anesthetized with sodium pentobarbital (JW Pharm Co, Ltd, Seoul, Korea, 40 mg/kg, ip) and perfused through the ascending aorta 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). Brain tissues containing the hippocampus were serially and frontally sectioned into 30-μm thickness in a cryostat (Leica, Wetzlar, Germany).

2.5. Immunohistochemistry

Immunohistochemistry were performed as previously described [21], briefly, the sections were treated with 0.3% hydrogen peroxide (H_2O_2) in PBS and 10% normal goat serum in 0.05 M PBS for 30 min, respectively. The treated sections were incubated with diluted rabbit *anti*-Ki-67 (1:1000, Abcam, UK), goat *anti*-DCX (1:50, Santa Cruz Biotechnology, USA), rabbit *anti*-BDNF (1:200, Abcam, UK), rabbit *anti*-TrkB (1:500, Santa Cruz Biotechnology, USA), rabbit *anti*-IGF-1 (1:200, Santa Cruz Biotechnology, USA), rabbit *anti*-IGF-IR (1:200, Santa Cruz Biotechnology, USA), and rabbit *anti-p*-CREB (1:250, Millipore, CA) as primary antibodies overnight at 4 °C. Thereafter the incubated sections were exposed to biotinylated goat anti-rabbit or rabbit anti-goat IgG (1:200, Vector, Burlingame, CA), and streptavidin peroxidase complex (1:200, Vector). Finally, the sections were visualized with 3,30-diaminobenzidine tetrahydrochloride in 0.1 M Tris–HCl buffer.

Negative control tests were carried out using pre-immune serum instead of each primary antibody to establish the specificity of each immunostaining. Each negative control resulted in the absence of immunoreactivity.

2.6. Double immunofluorescence staining

Double immunofluorescence staining for BrdU and NeuN was performed to observe differentiation from newly generated cells to mature neurons as previously described [22]. The sections were incubated in 50% formamide/2X SSC (0.3 M NaCl, 0.03 M sodium citrate) for 2 h at 65 °C, incubated in 2 N HCl for 30 min at 37 °C, and rinsed in 0.1 M boric acid (pH 8.5) for 10 min for BrdU immunostaining to visualize BrdU-labeled nuclei. Next, the rinsed sections were incubated in a mixture of rat *anti*-BrdU (1:100, BioSource International, Camarillo, USA) and rabbit *anti*-NeuN (1: 500, Chemicon, USA) overnight at 4 °C. Finally, the reacted sections were incubated in a mixture of both FITC-conjugated anti-rat IgG (1:200; Jackson ImmunoResearch, PA) and Cy3-conjugated anti-rabbit IgG (1:500; Jackson ImmunoResearch) for 2 h at room temperature. Double immunoreaction was observed under a confocal MS (LSM510 META NLO, Carl Zeiss, Germany).