3-[3-(3-florophenyl-2-propyn-1-ylthio)-1, 2, 5-thiadiazol-4-yl]-1, 2, 5, 6-tetrahydro-1-methylpyridine oxalate, a novel xanomeline derivative, improves neural cells proliferation and survival in adult mice

Xiaoliang Zhang¹, Qiang Gong², Shuang Zhang³, Lin Wang², Yinghe Hu¹, ², Haiming Shen³, Suzhen Dong¹, ²

¹Key Laboratory of Brain Functional Genomics, Ministry of Education, East China Normal University, Shanghai 200062, China
²Shanghai Engineering Research Center of Molecular Therapeutics and New Drug Development, Institute for Advanced Interdisciplinary Research, East China Normal University, Shanghai 200062, China
³Institute of Aviation Medicine, Civil Aviation University of China, Tianjin 300300, China

Abstract
The present study analyzed the influence of 3-[3-(3-florophenyl-2-propyn-1-ylthio)-1, 2, 5-thiadiazol-4-yl]-1, 2, 5, 6-tetrahydro-1-methylpyridine oxalate (EUK1001), a novel xanomeline derivative of the M4/M5 receptor agonist, on hippocampal neurogenesis in adult C57BL6 mice. Results showed that 15-day EUK1001 treatment via intraperitoneal injection promoted neural cell proliferation in the dentate gyrus, although cell differentiation did not change. The majority of bromodeoxyuridine-positive cells co-expressed the immature neuronal marker doublecortin. In addition, the level of neurogenesis in the subventricular zone was not altered. Brain-derived neurotrophic factor mRNA expression was up-regulated following EUK1001 treatment, but no change was observed in expression of camp-responsive element binding protein 1, paired box gene 6, vascular endothelial growth factor alpha, neurogenic differentiation factor 1, and wingless-related mouse mammary tumor virus integration site 3A mRNA. These experimental findings indicated that EUK1001 enhanced proliferation and survival of hippocampal cells, possibly by increasing brain-derived neurotrophic factor expression.

Key Words: EUK1001; brain-derived neurotrophic factor; M4/M5 receptor; neural regeneration; proliferation; survival

INTRODUCTION
The neurotransmitter acetylcholine is involved in many physiological functions, including synaptic plasticity, learning, and memory. Cholinergic receptors are classified into two types: nicotinic acetylcholine receptors and metabotropic muscarinic acetylcholine receptors. The M4 receptor is the most densely expressed metabotropic muscarinic receptor in the hippocampus and forebrain[1]. A number of pharmacological and pathological studies have linked M1 activity to cognitive function[2, 3]. Indeed, the loss of basal forebrain cholinergic neurons is associated with memory deficits in Alzheimer’s disease (AD) patients[4]. Cholinergic drugs that inhibit acetylcholinesterase or directly activate M1 muscarinic receptors provide potential treatment strategies for AD[5, 6]. In addition, the use of the M4/M5 receptor agonist, xanomeline, has been studied as a treatment candidate for AD[7, 8]. Although results from a large-scale, placebo-controlled, clinical trial demonstrated dose-dependent improvement in cognitive function and behavioral performance in AD patients, the development of xanomeline as a drug was suspended because of intolerable muscarinic side effects[9]. Our research group has synthesized a novel xanomeline derivative, 3-[3-(3-florophenyl-2-propyn-1-ylthio)-1, 2, 5-thiadiazol-4-yl]-1, 2, 5, 6-tetrahydro-1-methylpyridine oxalate (EUK1001), which has been shown to improve learning and memory, as well as enhance hippocampal synaptic plasticity, in aged mice[10, 11]. In addition, EUK1001 is less toxic than xanomeline[12] and alleviates AD-like neurodegenerative phenotypes in a preseminil-deficient mouse model[13]. Therefore, EUK1001 could prove to be a promising therapeutic agent for the treatment of AD and age-related cognitive decline. In contrast to these well-described
behavioral responses, the underlying molecular mechanisms of EUK1001-related cognitive enhancement remain poorly understood. Previous studies have shown that pharmacological blockade of muscarinic acetylcholine receptors impairs adult neurogenesis\cite{14-15}, but muscarinic receptor agonist administration increases neural cell proliferation in the subgranular zone (SGZ) and subventricular zone (SVZ)\cite{15-16}. In addition, acetyl cholinesterase inhibition following systemic donepezil treatment increases survival of newborn neurons in mice, but not proliferation of neural progenitor cells in the SGZ or SVZ\cite{21}. Furthermore, nicotinic acetylcholine receptors were shown to regulate hippocampal cell proliferation in adult mice\cite{18}. Results from these studies suggest that the cholinergic system plays an important role in regulating adult neurogenesis. However, the effects of muscarinic receptor activation by EUK1001 on adult neurogenesis remain to be determined.

Adult neurogenesis, in particular hippocampal neurogenesis, correlates with cognitive function. In different strains mice, a correlation between hippocampal neurogenesis and spatial memory has been established\cite{19}. In addition, oral administration of the cognitive enhancer spiro[imidazo [1, 2-a]pyridine-3, 2-indan]-2(3H)-one, which stimulates acetylcholine release, was demonstrated to promote hippocampal neurogenesis\cite{20}. In addition, cognitive dysfunction following inhibition of adult neurogenesis suggests that adult-born neurons continue to make significant contributions to cognitive function throughout life\cite{21}, including roles in pattern separation and system consolidation\cite{22}. Conversely, hippocampus-dependent learning enhances SGZ neurogenesis\cite{19}. Although the role of adult hippocampal neurogenesis in cognitive function remains elusive, growing evidence suggests it contributes to hippocampus-dependent memory. Based on known effects of EUK1001 on learning and hippocampal synaptic plasticity in mice, as well as the relationship between cognitive function, plasticity, and neurogenesis, it was speculated that EUK1001 treatment could affect neurogenesis in adult mice. To test this hypothesis, adult neurogenesis in C57BL6J mice was analyzed following EUK1001 administration.

**RESULTS**

**Quantitative analysis of experimental animals**

A total of 50 male, C57BL6J mice were included in the present study. Among them, 40 mice were randomly assigned to four neurogenesis groups, with ten mice in each group. Two of these groups were included in the cell proliferation experiment. Nine mice of each group were used in the experiment. The remaining two groups were included in the newborn cell survival experiment. In the cell survival experiment, six mice per group were chosen at random for experimentation. In the gene expression analysis experiments, ten mice were used. In total, 40 mice were included in the final analysis.

**EUK1001 treatment promoted hippocampal cell proliferation in mice** (Figure 1)

![Figure 1](image)

(A-F) Representative images of bromodeoxyuridine (BrdU) immunofluorescence and DAPI staining (A-C: EUK1001; D-F: control; scale bar: 100 μm). The large pink nucleus on the upper right of C shows a BrdU-labeled nucleus merged with DAPI, which is a magnification (× 10) of the nucleus identified by the arrowhead. Dentate gyrus contains all regions of the granule cell layer (GCL), subgranular zone (SGZ), and the hilus, as indicated in B. DAPI: 4', 6-diamidino-2-phenylindole.

(G) Statistical analysis of newborn cell numbers in the SGZ and dentate gyrus of EUK1001 and control groups. The number of BrdU-positive cells is presented as mean ± SEM. *P < 0.01, vs. control group; n = 9 in each group.
Brain slices from EUK1001-treated and saline-treated mice were subjected to immunofluorescence analysis of bromodeoxyuridine (BrdU). The average number of BrdU-positive (BrdU⁺) cells in the SGZ and dentate gyrus of the EUK1001-treated group was greater than in the saline-treated group. Results demonstrated that EUK1001 treatment induced an increase of hippocampal cell proliferation (P < 0.01; Figure 1). However, EUK1001 injection did not show any obvious effect on neural cell proliferation in the SVZ (supplementary Figure 1 online).

To determine the effect of EUK1001 treatment on neuronal differentiation, co-immunolabeling experiments were performed. The number of BrdU⁺ cells co-labeled with an antibody specific for the immature neuronal marker doublecortin (DCX) was quantified. Although EUK1001 treatment induced a significant increase in the number of newborn cells, it did not significantly alter the phenotype of these cells; the majority of BrdU⁺ cells co-expressed DCX (Figure 2).

**EUK1001 treatment enhanced survival of newborn neural cells in the hippocampus**
The majority of hippocampal progenitors in adult animals die before differentiation and functional integration\(^{23}\). To assess survival of the newly generated cells, mice were injected with BrdU on the first 2 days of drug or vehicle treatment and were sacrificed 3 weeks later. Three-week survival of newborn neural cells was assessed by BrdU immunohistochemistry. As revealed by BrdU labeling, a greater number of newborn cells survived in the SGZ of EUK1001-treated mice compared with vehicle-treated mice. These results suggested that EUK1001 administration induced an increase in three-week survival of newly generated cells in the hippocampus of adult mice (P < 0.01; Figure 3).

**EUK1001 treatment up-regulated hippocampal brain-derived neurotrophic factor (BDNF) expression**
Adult neurogenesis is regulated by a variety of extrinsic and intrinsic factors within the local micro-environment\(^{23}\). Expression of six genes linked to regulation of adult neurogenesis was measured by real-time PCR. Results showed increased expression of BDNF in the hippocampus after EUK1001 treatment (P < 0.05; Figure 4). Expression of all other candidate genes, including camp-responsive element binding protein 1, paired box gene 6, vascular endothelial growth factor alpha, neurogenic differentiation factor 1, and wingless-related mouse mammary tumor virus integration site 3A did not show any significant changes (supplementary Figure 2 online).
DISCUSSION

Previous studies have shown that a derivative of xanomeline, EUK1001, improves cognitive function and enhances hippocampal synaptic plasticity in old mice without significant toxicity\textsuperscript{[10-12]}. The present study analyzed the effects of this compound on adult neurogenesis. EUK1001 treatment for fifteen days resulted in significantly increased hippocampal cell proliferation and enhanced survival of these newly generated cells. The parent compound, xanomeline, is a potent functional agonist for M\textsubscript{1} and M\textsubscript{4} receptors\textsuperscript{[25]}, although it shows similar binding potency to M\textsubscript{2}\textsuperscript{[26]}, M\textsubscript{3}\textsuperscript{[27]}, and M\textsubscript{5} receptors (as a functional antagonist)\textsuperscript{[28]}. Therefore, EUK1001 might affect neurogenesis by affecting other muscarinic receptor subtypes, in addition to M\textsubscript{1}/M\textsubscript{4} receptors. However, M\textsubscript{1} and M\textsubscript{4} receptors are highly expressed in hippocampal granule neurons of the dentate gyrus\textsuperscript{[29]}. In addition, newly generated cells express M\textsubscript{1} or M\textsubscript{4} receptor\textsuperscript{[10]}. Therefore, it is possible that EUK1001 promotes hippocampal cell proliferation via M\textsubscript{1}/M\textsubscript{4} receptor stimulation. The effects of EUK1001 on hippocampal long-term potentiation are blocked by pirenzepine, a selective M\textsubscript{1} antagonist, but not by the selective M\textsubscript{2}/M\textsubscript{4} antagonist methoctramine\textsuperscript{[10-11]}, suggesting that EUK1001 affected hippocampal long-term potentiation via M\textsubscript{1} receptor stimulation. Recent studies have shown that adult neurogenesis is also involved in the regulation of hippocampal long-term potentiation\textsuperscript{[30]}, which suggests that the effects of EUK1001 could also be mediated via M\textsubscript{1} receptor stimulation. Further studies are needed to determine the effects of M\textsubscript{1} receptor blockade on hippocampal neurogenesis. Activation of M\textsubscript{1}/M\textsubscript{4} receptors by small molecular agonists can induce neurogenesis in the hippocampus, which is consistent with a recent study showing that pharmacological activation of M\textsubscript{1} receptors enhances...
SGZ neural cell proliferation in normal mice and mice with deficits in cholinergic function\(^{[16]}\). Degeneration of basal forebrain cholinergic neurons and deficient hippocampal neurogenesis has been observed in several AD animal models\(^{[31]}\). Therefore, sustained muscarinic activation may be therapeutically beneficial in AD patients. Our group recently showed that EUK1001 improves progressive neurodegenerative phenotypes in presenilin double knock-out mice\(^{[13]}\).

In the present study, EUK1001 treatment induced a robust increase in newly generated cells and enhanced their 3-week survival, as determined by BrdU-labeling during the early days of EUK1001 treatment. However, this compound did not alter neuronal differentiation; most of the BrdU\(^+\) neurons expressed the immature neuronal marker DCX. Interestingly, muscarinic activation did not alter SVZ neurogenesis, suggesting different mechanisms underlying adult neurogenesis in the SGZ and SVZ\(^{[24]}\).

Adult hippocampal neurogenesis is regulated by myriad neurotransmitters, hormones, growth factors, and transcription factors\(^{[24]}\). To determine the possible mechanisms associated with EUK1001-evoked neurogenesis, expression of six genes related to neuronal proliferation and survival was analyzed. Real-time quantitative PCR analysis demonstrated BDNF expression significantly increased in the hippocampus after EUK1001 treatment, and BDNF has been previously shown to promote neuronal cell proliferation in the dentate gyrus\(^{[32]}\). The effect of EUK1001 on induction of BDNF expression might be involved in hippocampal neurogenesis.

Results from the present study demonstrated that EUK1001 treatment enhanced hippocampal cell proliferation and newborn cell survival.

**MATERIALS AND METHODS**

**Design**
A randomized, controlled experiment.

**Time and setting**
The present study was performed at the Key Laboratory of Brain Functional Genomics, Ministry of Education, East China Normal University, China from September 2009 to September 2010.

**Materials**

**Animals**
Eight-week-old, male, C57BL6J mice, weighing 20 ± 4 g, were used in these studies. All mice were maintained at 22 ± 3°C under 40–70% humidity, with a 12-hour light/dark cycle. The mice were allowed free access to standard chow and water. All procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China\(^{[33]}\).

**Drugs**
EUK1001 was synthesized at the College of Medicine, Peking University, China. Its chemical structure is shown in Figure 5.

![Figure 5: The chemical structural formula of xanomeline (A) and 3-\{3-(3-florophenyl-2-propyn-1-ythio)-1, 2, 5-thiadiazol-4-yl\}-1, 2, 5, 6-tetrahydro-1-methylpyridine oxalate (B)\(^{[10]}\).](image)

**Methods**

**EUK1001 administration**
For the cell proliferation experiment, mice received a daily intraperitoneal injection of EUK1001 in sterile 0.9% NaCl solution at a dose of 1 mg/kg (EUK1001 group) or an equal volume of sterile 0.9% NaCl solution (control group) for 15 consecutive days. The dose was chosen based on previous studies\(^{[11]}\), as well as our preliminary studies. On the last two days of EUK1001 or vehicle injection, the mice were injected with BrdU (10 mg/mL in sterile 0.9% NaCl solution, 100 mg/kg; Sigma, St. Louis, MO, USA) four times per day at 2-hour intervals. The mice were perfused and sacrificed on day 15 after the last injection.

For the cell survival experiment, mice were injected with BrdU or vehicle at the same dose on the first 2 days of EUK1001 administration, and were sacrificed 3 weeks later. The BrdU labeling paradigm was performed as previously described\(^{[33]}\), with minor modifications. The dose was chosen based on previous studies\(^{[35]}\).

In the real-time PCR experiments, mice from EUK1001 and control groups (five each group) were treated with 1 mg/kg EUK1001 or saline, respectively, for 15 days and were sacrificed one day later.

**Tissue preparation**
The mice were deeply anaesthetized with pentobarbital (50 mg/kg) and transcardially perfused with sterile 0.9% NaCl solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The brains were removed and immediately fixed in 4% paraformaldehyde-PBS for 2 hours, then sequentially immersed in 20% and 30% sucrose-PBS, respectively, until the brains sank. Subsequently, 30-μm thic coronal sections were cut on a freezing microtome (Leica, Wetzlar, Germany). Sections between the anterior landmark, where the infrapyrmidal and suprapyrmidal...
blade of the dentate gyrus granule cell layer formed (roughly −1.34 mm lateral to bregma), and the posterior landmark, where the dorsal dentate gyrus connects to the ventral region (about −2.92 mm lateral to bregma)[14], were mounted on pre-treated slides (Premiere, New York, NY, USA) (60 slices/mouse) and stored at −80°C until further use.

**Immunofluorescence detection of BrdU and microtubule-associated protein**

BrdU immunofluorescence was performed as previously described[30]. Briefly, sections were washed three times in 0.1 M PBS (pH 7.4)/0.1% Triton-X100 for 5 minutes each. Antigen retrieval was performed by incubating the sections in 10 mM sodium citrate buffer (pH 6.0) at 92–98°C for 15 minutes. After cooling for 20 minutes in the same buffer, the sections were rinsed for 5 minutes in 0.1 M PBS/0.1% Triton-X100. The sections were incubated in blocking solution (5% goat serum, 3% Triton-X100, and 3% bovine serum albumin in 0.1 M PBS) for 60 minutes at room temperature, followed by mouse anti-BrdU primary antibody (1: 5 000; Millipore, MA, USA) and rabbit anti-DCX polyclonal antibody (1: 500; Abcam, Cambridge, UK) overnight at 4°C. After rinsing six times in 0.1 M PBS Tween-20 (0.1% Tween in 0.1 M PBS, pH 7.4) for 5 minutes each on a shaker, the sections were incubated in Cy3-labeled goat anti-mouse secondary antibody (1: 400; Millipore) and Alexa488-labeled goat anti-rabbit secondary antibody (1: 1 000; Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. The sections were thoroughly washed six times in 0.1 M PBS Tween-20 for 5 minutes each on a shaker. Finally, the sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted on coverslips with anti-fading mounting medium (Beyotime, Jiangsu Province, China). Fluorescence signals were detected with a Nikon microscope (Nikon, Tokyo, Japan). Boundaries of the granule cell layer (GCL) (dentate gyrus, and hilus) were identifiable by DAPI staining and digitally outlined. The SGZ was defined as a two-cell body wide zone along the border of the GCL and the hilus[37]. All BrdU-positive cells within the outlined areas from every fifth section were quantified at 400 × magnification. The GZ and dentate gyrus area from each selected section was measured in Photoshop software (Adobe, California, CA, USA), and the average total area was calculated. The number of BrdU cells in the dentate gyrus was calculated as the sum of BrdU cells in the GCL, SGZ, and hilus. The average number of BrdU-labeled cells/mm² in the SGZ and dentate gyrus/section was calculated.

**Real-time PCR detection of BDNF, camp-responsive element binding protein 1, paired box gene 6, vascular endothelial growth factor alpha, neurogenin differentiation factor 1, and wingless-related mouse mammary tumor virus integration site 3A mRNA expression in the hippocampus**

Male mice were decapitated, and the hippocampi were rapidly dissected, pooled, and stored at −80°C until further use. Total RNA was extracted from the frozen tissues using Trizol (Invitrogen, Carlsbad, CA, USA). The RNA samples were used to generate cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA samples were used as templates for SYBR Green qRT-PCR. The primers were designed using PrimerExpress software (Applied Biosystems, Foster City CA, USA) and synthesized by Sangon (Shanghai, China). Primer sequences for BDNF amplification were 5’TCA CCA GGA TCT AGC CGC CG’ and 5’CGT CAT GCA ACC GAA-3’, which targeted to the first and second exons of BDNF transcript variant 2 (NM_001048139). The remaining primer sequences are listed in supplementary Table 1 online. Glyceraldehyde-3-phosphate dehydrogenase was used as the internal reference gene. The experiment was performed using Opticon 2 (BioRad, Hercules, CA, USA) as previously described[38]. Gene expression levels were calculated and expressed as 2−ΔΔCt values.

**Statistical analysis**

Data are presented as mean ± SEM. Statistical significance was determined by two-tailed Student’s t-test with Sigmastat 10.0 Software (Systat Software, San Jose, CA, USA), and P < 0.05 was considered statistically significant.

**Author contributions:** Suzhen Dong and Yinghe Hu conceived and designed the experiments. Xiaoliang Zhang and Qiang Gong performed animal and immunohistological experiments. Shuang Zhang and Lin Wang performed the cell quantifications. Suzhen Dong analyzed the data and wrote the manuscript. Haiming Shen assisted in experimentation.

**Conflicts of interest:** None declared.

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**Ethical approval:** All experimental procedures were approved by the East China Normal University Committee for the Care and Use of Laboratory Animals, China.

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**Supplementary information:** Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol. 7, No. 1, 2012 item after selecting the “NRR Current Issue” button on the page.

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