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Research Paper

Anti-Depressant Fluoxetine Reveals its Therapeutic Effect Via Astrocytes

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ABSTRACT

Although psychotropic drugs act on neurons and glial cells, how glia respond, and whether glial responses are involved in therapeutic effects are poorly understood. Here, we show that fluoxetine (FLX), an anti-depressant, mediates its anti-depressive effect by increasing the gliotransmission of ATP. FLX increased ATP exocytosis via vesicular nucleotide transporter (VNUT). FLX-induced anti-depressive behavior was decreased in astrocyte-selective VNUT-knockout mice or when VNUT was deleted in mice, but it was increased when astrocyte-selective VNUT was overexpressed in mice. This suggests that VNUT-dependent astrocytic ATP exocytosis has a critical role in the therapeutic effect of FLX. Released ATP and its metabolite adenosine act on $P2Y_{11}$ and adenosine A2b receptors expressed by astrocytes, causing an increase in brain-derived neurotrophic factor in astrocytes. These findings suggest that in addition to neurons, FLX acts on astrocytes and mediates its therapeutic effects by increasing ATP gliotransmission.

1. Introduction

Depression is a major public health problem worldwide. About 350 million people suffer from the disease and it will be ranked the second leading cause of death by the year 2020 [49]. There are several effective treatments for depression, but it is estimated that one-third of depressed patients do not respond adequately to conventional antidepressant drugs. Moreover, the slow onset of their therapeutic effects also restricts antidepressant use. Thus, there is an urgent need to identify the biological mechanism of depression and the pharmacological action of antidepressants. It is thought that antidepressants mediate their therapeutic effects by acting on neurons especially monoaminergic neurons, but they also act on non-neuronal cells such as glial cells. However, to date, how glial cells respond to antidepressants or whether glial responses are involved in the therapeutic effects of antidepressants remains unknown.

Astrocytes are the most abundant glial cells in the brain. In addition to their classical roles such as providing physical support to neurons or the removal of neuronal waste, astrocytes are active regulators of brain functions by releasing so-called "gliotransmitters" such as ATP, glutamate and D-serine [26]. Of these, ATP has received increased attention because it is released from astrocytes [25] and mediates various functions to regulate adjacent cells. In addition, released ATP is metabolized into adenosine, and both ATP and adenosine provide autocrine and paracrine signals via P2 and P1 receptors, respectively. Regarding the release of ATP, multiple pathways were reported, including connexin hemi-channels [16], pannexin hemi-channels [61], maxi-anion channels [40], $P2X_7$ receptors [60] and exocytosis. Recently, Sawada et al. [51] reported vesicular nucleotide transporter (VNUT) uptakes ATP into intracellular vesicles. ATP was released by VNUT-dependent exocytosis in several types of cells including neurons [41], keratinocytes [30], microglia [29] and astrocytes [24, 35]. Astrocytic ATP has gained increasing attention because a recent report by Cao et al. clearly showed that decreased extracellular ATP mediated by astrocytes in the hippocampus caused depression in mice [6]. However, the mechanisms underlying the contribution of decreased ATP to depressive behavior, and whether anti-depressants affect astrocytic ATP functions, are poorly understood.

Brain-derived neurotrophic factor (BDNF) is increased by antidepressants and is considered to have a major role in the therapeutic action of antidepressants. For example, reduced BDNF levels were reported in depressed patients and models of depression, and antidepressant treatment increased BDNF expression [21]. It is well known that the majority of BDNF is produced by neurons [42] as well as microglial

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cells [28]. BDNF levels are very low in astrocytes from normal healthy adult brains, although other neurotrophic factors (glial cell–derived neurotrophic factor (GDNF) and nerve growth factor (NGF)) are synthesized in astrocytes [50]. However, BDNF was increased in response to changes in brain environment such as increased ATP [62], suggesting that astrocytes might be a source of BDNF during certain circumstances.

In the present study, we demonstrated that an antidepressant, fluoxetine (FLX), acts on astrocytes to mediate anti-depressive effects in mice. We also show that FLX increases extracellular ATP via VNUT, which subsequently increases BDNF in astrocytes. Thus, astrocytes and its related molecules depression are of great interest to understand further the therapeutic effects of FLX.

2. Materials and Methods

2.1. Animals

All experiments were carried out in accordance with the "Guiding Principles in the Care and Use of Animals in the Field of Physiologic Sciences" published by the Physiologic Society of Japan (LI, 2002) and with the approval of the Animal Care Committee of Yamanashi University (Chuo, Yamanashi, Japan). C57BL/6J mice (17-day-old fetuses or 9 week-old males) and Wistar rats (17-day-old fetuses) were obtained from Japan SLC (Shizuoka, Japan).

2.2. Generation of Mlc1-tTS BAC Transgenic Mice

The codons of bacterial tetracycline activator protein and human zinc finger protein KRAB domain were fully mammalianized (tTS). Mouse BAC DNA (clone RP23-114I6) was initially modified by inserting a Rpsl-Zeo cassette (gift from Dr. Hisashi Mori) into the translation initiation site of the *Mlc1* gene followed by replacement with a cassette containing tTS and SV40 polyadenylation signal. BAC DNA was linearized by PI-*Sce*I (Cat. # R0696S, New England Biolabs Inc., Massachusetts, U.S.A) enzyme digestion, and injected into fertilized eggs from CBA/C57BL6 mice.

2.3. Generation of VNUT-tetO Knock-in Mice

tetO responsive transgenes were constructed by placing a tetO responsive promoter element by use of 129 SvEv ES cells (Cat. # CMTI-1, RRID:CVCL_GS41). The tetO sequence was inserted upstream of the translation initiation site, and tetO insertion did not alter wild-type expression patterns [64]. Therefore, VNUT protein levels in VNUT-tetO homozygous mice were equivalent to those in wild-type mice.

2.4. Doxycyline-Mediated Control of Gene Expression in Double Transgenic Mice

We did not administer doxycycline to inhibit tTA- or tTS-mediated transcriptional control.

2.5. Generation of Astro-VNUT-KO and Astro-VNUT-OE Mice

We crossed *Mlc1*-tTS or *Mlc1*-tTA BAC transgenic mice with VNUT-tetO knock-in mice to generate Mlc-tTS::VNUT-tetO homozygous mice (astro-VNUT-KO), Mlc-tTA::VNUT-tetO homozygous mice (astro-VNUT-OE) and VNUT-tetO homozygous mice as littermate controls.

All mice were housed in plastic cages in groups of one to five per cage, at room temperature, and with free access to water and food. They were kept on an artificial 12 h light/dark cycle.

2.6. Experimental Schedule for Drug Treatment of Mice

FLX was freshly dissolved in saline before use. Animals were administered with FLX orally at a dose of 10 or 20 mg/kg or saline, using a volume of 10 ml/kg once daily for 21–28 days.

2.7. Measurement of Extracellular ATP in the Hippocampus

A previously described procedure for tissue ATP measurement [6] was used with some modifications. Briefly, mice were deeply anesthetized with pentobarbital and the hippocampal tissues were removed immediately. Transverse slices (300 μm thick) from the hippocampus were prepared using a tissue slicer (D.S.K. LINEARSLICER PRO7). Slices were immersed for 18 min in bubbled artificial cerebrospinal fluid (ACSF) composed of 125 mM NaCl, 5.0 mM KCl, 2.0 mM $CaCl_2$, 2.0 mM $MgSO_4$, 10 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 10 mM D-glucose and the ectonuclease inhibitor ARL67156 (100 mM) (95% oxygen and 5% carbon dioxide; 4 °C). Then the ACSF was collected and ATP levels were measured using an ATP determination kit (ATP Bioluminescence Assay Kit CLS II; Cat. # 11699695001, Roche Applied Science, Basel, Switzerland). Luminescence was measured by a luminometer (Berthold Lumat LB 9501). For normalization, protein amounts of each sample were measured by the bicinchoninic acid assay (Thermo Fisher Scientific, USA).

2.8. Tail Suspension Test

Animals were tested using a modified version of the tail suspension test (TST) that has been previously validated [58]. On the testing day, mice were brought into the behavior room 1 h before the test session to allow them to habituate to the environment. All experimental testing sessions were conducted between 12:00 P.M. and 6:00 P.M., with animals assigned and tested randomly. Eight FLX-treated animals were used, with a matched number of saline-treated control subjects. Each behavioral test was conducted 1 h after the previous drug injection. Mice were individually suspended by the tail with clamp (1 cm distant from the end) for 6 min in a box (MSC2007, YTS, Yamashita Giken, Tokushima, Japan) with the head 10 cm above the bottom of the box. Testing was carried out in a darkened room with minimal background noise. The duration of immobility was scored manually during a 6 min test. The behavioral measure scored was the duration of "immobility", defined as the time when the mouse did not show any movement of the body and hanged passively.

2.9. Immunohistochemistry

After perfusion, brain segments were postfixed in 4% paraformaldehyde for 24 h, and then permeated with 20% sucrose in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for 24 h and 30% sucrose in 0.1 M PBS for 48 h at 4 °C. Brain segments were frozen in an embedding compound (Sakura Finetek, Tokyo, Japan) on dry ice. They were cut with a cryostat (Leica CM 1100; Leica, Wetzlar, Germany) at a thickness of 30 μm and collected in PBS at 4 °C to be processed immunohistochemically as free-floating sections. The sections were incubated for 48 h at 4 °C with primary antibodies: mouse anti-GFAP (1:2000; Cat. # AB5804, RRID: AB_10062746), rabbit anti-BDNF (1:2000; Cat. # sc-546, RRID:AB_630940). The sections were washed six times with 0.1 M PBS (10 min each) and then incubated for 3 h at room temperature with the secondary antibody: Alexa488- and Alexa546-conjugated mouse- and rabbit-IgGs.

(Cat. # A-11034, RRID:AB_2576217 and Cat. # A11030, RRID: AB_144695). Immunohistochemical images were obtained using a confocal laser microscope (Fluoview1000; Olympus, Tokyo, Japan) and digital images were captured with Fluoview1000 (Olympus).

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