

Article

L-Dopa and Fluoxetine Upregulate Astroglial 5-HT_{2B} Receptors and Ameliorate Depression in Parkinson's Disease Mice

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Abstract: Here, we report the association between depressive behavior (anhedonia) and astroglial expression of 5-hydroxytryptamine receptor 2B (5-HT_{2B}) in an animal model of Parkinson's disease, induced by bilateral injection of 6-hydroxydopamine (6-OHDA) into the striatum. Expression of the 5-HT_{2B} receptor at the mRNA and protein level was decreased in the brain tissue of 6-OHDA-treated animals with anhedonia. Expression of the 5-HT_{2B} receptor was corrected by four weeks treatment with either L-3,4-dihydroxyphenylalanine (L-dopa) or fluoxetine. Simultaneously, treatment with L-dopa abolished 6-OHDA effects on both depressive behavior and motor activity. In contrast, fluoxetine corrected 6-OHDA-induced depression but did not affect 6-OHDA-induced motor deficiency. In addition, 6-OHDA downregulated gene expression of the 5-HT_{2B} receptor in astrocytes in purified cell culture and this downregulation was corrected by both L-dopa and fluoxetine. Our findings suggest that 6-OHDA-induced depressive behavior may be related to the downregulation of gene expression of the 5-HT_{2B} receptor but 6-OHDA-induced motor deficiency reflects, arguably, dopamine depletion. Previously, we demonstrated that fluoxetine regulates gene expression in astrocytes by 5-HT_{2B} receptor-mediated transactivation of epidermal growth factor receptor (EGFR). However, the underlying mechanism of L-dopa action remains unclear. The present work indicates that the decrease of gene expression of the astroglial 5-HT_{2B} receptor may contribute to development of depressive behavior in Parkinson's disease.

Keywords: 5-HT_{2B} receptor; astrocytes; depressive behavior; Parkinson's disease; fluoxetine

1. Introduction

Parkinson's disease (PD) is characterized by a progressive degeneration of dopaminergic midbrain neurons in the substantia nigra pars compacta (SNpc) [1] and becomes clinically manifest when more than 50% of SNpc neurons are lost. In addition to motor symptoms, which include resting tremor, slowness of movement, rigidity, and postural instability, the non-motor symptoms, such as cognitive deficits and behavioral abnormalities, have been recognized as integral part of the clinical presentation of PD [2]. Depression is the frequent psychiatric signature of the PD and it is one of the most significant factors affecting the quality of life of patients [3]. Whether L-3,4-dihydroxyphenylalanine (L-dopa) treatment improves depression in PD is controversial (see [4] for review). Nevertheless, the serotonergic



system is affected in PD patients and in some animal models [4], while 5-hydroxytryptamine (5-HT) depletion may contribute to motor and non-motor symptoms of PD [5].

The role of astroglia in pathological evolution of PD remains to be revealed in detail, although both astroglial reactivity and astrodegeneration with loss of function and compromised neuroprotective capacity are documented [6,7]. It is generally acknowledged that the morbid changes associated with depression include profound remodeling of neuroglia, and furthermore, the contribution of astrocytes to the pathogenesis of various neuropsychiatric disorders is well appreciated [8,9]. Previously, we reported that fluoxetine, a selective serotonin reuptake inhibitor (SSRI) activates astroglial 5-hydroxytryptamine receptor 2B (5- HT_{2B}) receptors which results in transactivation of epidermal growth factor receptor (EGFR) [10]. We also found that expression of 5-HT_{2B} receptors as well as other signaling molecules is suppressed in astrocytes but not in neurons in the cerebral cortex of anhedonic animals, which experienced chronic mild stress (CMS) [11,12]. Chronic treatment with fluoxetine eliminated both decrease in expression of astroglial 5-HT_{2B} receptors and anhedonia [11]. These findings corroborate the role for astrocytic 5-HT_{2B} receptor in depressive behavior. Recently, we also found a decrease in gene expression of astrocytic but not neuronal 5-HT_{2B} receptor in animals that received 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and became anhedonic. Fluoxetine corrected MPTP-induced decrease of 5-HT_{2B} receptor expression and depressive behavior [13]. These findings indicate that changes in gene expression of 5-HT_{2B} receptors in astroglia may be associated with pathophysiological evolution of depression in PD.

Another animal model of PD is induced by a bilateral injection of 6-hydroxydopamine (6-OHDA) into the striatum. 6-Hydroxydopamine has high affinity to dopamine transporter and the structure of 6-OHDA is similar to that of dopamine, but the presence of an additional hydroxyl group makes it toxic to dopaminergic neurons [14]. Injection of 6-OHDA into the striatum induces retrograde degeneration of tyrosine hydroxylase (TH)-positive terminals in the striatum which instigated death of TH positive neurons in the SNpc, similarly to PD in humans [14]. Using the 6-hydroxydopamine animal model of PD, this study aimed to examine effects of L-dopa and fluoxetine on the gene expression of the 5-HT_{2B} receptor in primary cultures of astrocytes and in the brain of animals treated with 6-OHDA and correlates these changes with the depressive behavior and motor deficits.

2. Materials and Methods

All experimental techniques were essentially similar to those employed in our previous studies of astroglial 5-HT_{2B} receptors [11–13]. All experiments were carried out in accordance with the USA National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023) and its 1978 revision, and all experimental protocols were preregistered and approved by the Institutional Animal Care and Use Committee of China Medical University.

2.1. Animals

CD-1 mice (Charles River, Beijing, China), weighing 30–40 g, male C57BL/6 mice (Chang Sheng Biotechnology, Benxi, China), weighing 22–26 g and mice with fluorescently tagged astrocytes and neurons (males FVB/NT-g(GFAP-GFP)14Mes/J or B6.Cg-Tg(Thy1-YFPH)2 Jrs/J, respectively; the Jackson Laboratory, Bar Harbor, ME, USA), weighing 20–25 g were kept at standard housing conditions with light/dark cycle of 12 h. Water and food were provided at libitum.

2.2. 6-OHDA Treatment

C57BL/6 mice were anesthetized with pentobarbital and mounted in a stereotaxic frame. Each mouse received a bilateral injection of 1 μ L 6-OHDA (5 μ g/ μ L in saline containing 0.02% ascorbic acid) into the dorsal-lateral striatum, according to the following coordinates (mm): antero-posterior + 0.5, medio-lateral \pm 2, and dorso-ventral -3. Control sham-lesioned mice were injected with the same volume of vehicle (saline). After surgery, the animals were allowed to recover for three weeks.

2.3. Drug Treatment

After three-week recovery from the 6-OHDA lesions, anhedonic mice were daily injected intraperitoneally with fluoxetine (10 mg/kg/d dissolved in saline) or L-dopa (20 mg/kg/d in saline and combined with 12 mg/kg/d of benserazide) for four weeks. In the present study, mice were separated into six groups: (1) sham-lesioned animals treated with saline (Control); (2) sham-lesioned animals treated with fluoxetine (Flu); (3) sham-lesioned animals treated with L-dopa (L-dopa); (4) 6-OHDA-lesioned animals treated with saline (6-OHDA); (5) 6-OHDA-lesioned animals treated with fluoxetine (6) (4) 6-OHDA + Flu); (6) 6-OHDA-lesioned animals treated with L-dopa (6) (4) 6-OHDA + L-dopa).

Prior to surgery, three and seven weeks thereafter, mice underwent behaviour tests for motor function and mood. After three weeks of 6-OHDA treatment, only mice with depressive behavior (around 60%) were selected for L-dopa or fluoxetine treatment for another four weeks. At the end of the experiments (seven weeks), mice were sacrificed and cerebral cortex was dissected out for gene expression analysis of 5-HT_{2B} receptor. FVB/NT-g(GFAP-GFP)14Mes/J or B6.Cg-Tg(Thy1-YFPH)2 Jrs/J mice were sacrificed after three-week recovery from the 6-OHDA lesions.

2.4. Behavioral Tests

Several behavioral tests were applied to 6-OHDA-treated mice to assess motor activity (pole test and rotarod test) and depression behavior (sucrose preference test, forced swim, tail suspension and open field tasks).

The pole test was performed as previously described [15] with minor modifications. The mouse was placed head-upward on the top of a vertical rough-surfaced pole (diameter 1 cm; height 55 cm). The time to turn downward from the top (T-turn time) and to descend to the floor (locomotor activity time, T-LA time) were measured. The total time was recorded with a maximum duration of 30 s.

Motor coordination was assessed with rotarod test. Mice were positioned on a rotating bar set to a rotation speed of up to 18 rpm during the test. The time spent on the rotating bar, known as the latent period, was recorded. Latency to fall was recorded with a stopwatch, with a maximum of 90 s. The test was repeated twice and mean latencies were analyzed.

The depressive behavior was assessed with despair-based tests represented by the tail suspension test and forced swimming test, as well as with anxiety-based open field test. In tail suspension test mice were individually suspended from their tails at the height of 20 cm using a piece of adhesive tape wrapped around the tail 2 cm from the tip. Behavior was videotaped for 6 min. The duration of immobility was measured by an observer blinded to the treatment groups. Mice were considered immobile only when completely motionless and mice that climbed their tails were excluded from the data.

In the forced-swimming test, animals were dropped into glass cylinders (20×20 cm) containing 20 cm deep water maintained at 25 ± 1 °C and kept in water for 6 min. The time of immobility was recorded during the last 4 min of the 6 min testing period, followed by 2 min of habituation.

In the open field test, mice were placed in the central square of the open field box ($60 \times 60 \times 40$ cm) divided in to nine squares. Behavior was videotaped for 5 min. The parameters used for analysis included number of squares crossed, frequency of rearing, and time spent in the central area.

The sucrose preference test is a reward-based test and provides a measure of anhedonia, the lack of interest in pleasant activities. Anhedonia is a characteristic symptom of major depression [11]. Baseline sucrose preference was measured before lesion. After 20 h of food and water deprivation, mice were placed in individual cages and presented with two pre-weighted bottles, one containing 2.5% sucrose solution and another filled with water for 2 h. Percent preference was calculated according to the following equation: % preference = (sucrose intake/(sucrose + water intake)) × 100. A decrease of sucrose preference below 65% was taken as the criterion for anhedonia. This criterion was based on the fact that none of the control mice exhibited less than or equal to 65% preference for sucrose at that time point of the experiment.

For identification of acutely isolated cells, we used transgenic mice expressing a fluorescent marker under control of a cell-specific promoter (glial fibrillary acidic protein (GFAP) for astrocytes or cell surface glycoprotein Thy1 for neurons) thus allowing fluorescence-activated sorting of specified cell fractions; for detailed description of the technique see [12,16,17]. After isolation, cells were sorted by fluorescence-activated cell sorting (FACS) using the BD FACSAria Cell Sorting System (35 psi sheath pressure, FACSDiva software S/W 2.2.1; BD Biosciences, San José, CA, USA). Cell identity and purity were verified by mRNA expression of cell markers of astrocytes, neurons, and oligodendrocytes,

analyzed by reverse-transcription polymerase chain reaction (RT-PCR), in astrocytic and neuronal cell preparations. As shown previously [17], there is no contamination with neuronal or oligodendrocytic genes in the samples of astrocytes or of astrocytic or oligodendrocytic genes in the neuronal samples.

2.6. Primary Cultures of Astrocytes

Primary cultures of mouse astrocytes were prepared from the neopallia of the cerebral hemispheres of newborn CD-1 mice as previously described [18,19], sparsely seeded and grown in Dulbecco's minimum essential medium (DMEM) with 7.5 mM glucose. After two weeks in vitro, 0.25 mM dibutyryl cyclic adenosine monophosphate (dBcAMP) was included in the medium. These cultures are highly enriched in astrocytes as assessed by GFAP and glutamine synthetase expression [20]. Incubation with dBcAMP promotes morphological and functional differentiation as evidenced by the extension of cell processes and increases in several metabolic and functional activities characteristic of astrocytes in situ [21]. 6-Hydroxydopamine at 20 μ M was added to the culture after three weeks of culturing and continued for 6, 12, 24, and 48 h.

2.7. Reverse Transcription-Polymerase Chain Reaction

For determination of the mRNA expression of the 5- HT_{2B} receptor by RT-PCR, all samples from the cerebral cortex or astrocyte cultures were homogenized in Trizol (Invitrogen, Carlsbad, CA, USA). The RNA pellet was precipitated with isopropanol, washed with 75% ethanol, and dissolved in 10 μ L sterile, distilled water and an aliquot was used for determination of the amount of RNA [22].

Reverse transcription was initiated by a 5 min incubation at 65 °C of 1 μ g RNA extract with Random Hexamer (TaKaRa, Daliang, China) at a final concentration of 12.5 ng/L and deoxy-ribonucleoside triphosphates (dNTPs) at a final concentration of 0.5 mM. The mixture was rapidly chilled on ice and briefly spun and 4 μ L 5 × First-Strand Buffer, 2 μ L 0.1 M dithiotreitol and 1 μ L RNaseOUT Recombinant RNase Inhibitor (40 U/ μ L) (TaKaRa) were added. After the mixture had been incubated at 42 °C for 2 min, 1 μ L (200 U) of Superscript II was added and the incubation at 42 °C continued for another 50 min. Subsequently the reaction was inactivated by heating to 70 °C for 15 min and the mixture was chilled and briefly centrifuged.

Polymerase chain reaction amplification was performed in a Robocycler thermocycler (Biometra, Westburg, The Netherlands) with 0.2 μ M of sense or antisense and 0.375 U of Taq polymerase for 5-HT_{2B} receptor (forward, 5'-CTCGGGGGTGAATCCTCTGA-3'; reverse, 5'-CCTGCTCATCACCCTCTCTCA-3') [22], for TATA box-binding protein (TBP), used as a housekeeping gene (forward, 5'-CCACGGACAACTGCGTTGAT-3'; reverse, 5'-GGCTCATAGCTACTGAACTG-3') [23]. Initially, the template was denatured by heating to 94 °C for 2 min, followed by 2.5 min amplification cycles, each consisting of two 45 s periods and one 60 s period, the first at 94 °C, the second at 61 °C for 5-HT_{2B} receptor and at 55 °C for TBP and the third at 72 °C. The final step was extension at 72 °C for 10 min. The PCR products were separated by 1% agarose gel electrophoresis, stained with 0.5 μ g/mL ethidium bromide, and captured by Fluorchem 5500 (Alpha Innotech Corporation, San Leandro, CA, USA). The sizes of the PCR product of 5-HT_{2B} receptor was 370 bp and that of TBP 236 bp.

2.8. Western Blotting

Protein content was determined by the Lowry method [24], using bovine serum albumin as the standard. Samples containing 50 µg protein were applied on slab gels of 10% polyacrylamide and electrophoresed. After transfer to polyvinylidene fluoride (PVDF) membranes, the samples were blocked by 5% skim milk powder in TBS-T (30 mM Tris-HCl, 125 mM NaCl, 0.1% Tween 20) for 1 h. The PVDF membranes were incubated with the primary antibody, specific to 5-HT_{2B} receptor overnight at 4 °C or β-actin for 2 h at room temperature. After washing, the blots were incubated with peroxidase-conjugated affinity-purified goat anti-rabbit or goat anti-mouse horseradish peroxidase (HRP) antibody for 2 h. Staining was visualized by enhanced chemiluminescence (ECL) detection reagents. Digital images were obtained using Gel-Imaging System (Tanon 4200, Shanghai, China). Optical density for each band was assessed using the Window AlphaEase TM FC 32-bit software (Genetic Technologies, Miami, FL, USA). Ratios were determined between scanned 5-HT_{2B} receptor and β-actin, the latter used as housekeeping protein.

2.9. Statistics

Differences between multiple groups were evaluated by two-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) multiple comparison test for unequal replications. The level of significance was set at p < 0.05.

2.10. Materials

Most chemicals, including 6-OHDA, fluoxetine, L-dopa, benserazide, DNase I, propidium iodide, 6,7-dinitroquinoxaline-2,3-dione (DNQX), 2-amino-5-phosphonovalerate (APV), and first antibodies and the first β-actin antibody were purchased from Sigma (St. Louis, MO, USA). BD Biosciences (Franklin Lakes, NJ, USA) supplied the first antibody, raised against 5-HT_{2B} receptor. The second antibody goat anti-mouse IgG HRP conjugate was from Promega (Madison, WI, USA) and goat anti-rabbit IgG HRP conjugate from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence detection reagents were from Amersham Biosciences (Buckinghamshire, UK). Random Hexamer, deoxyribonucleotide triphosphates (dNTPs) and Taq-polymerase for RT-PCR were purchased from TaKaRa Biotechnology Co., Ltd. and Superscript II from Gibco Life Technology Invitrogen (Grand Island, NY, USA). Chemicals for preparation of culturing medium were purchased from Sigma and horse serum from Invitrogen.

3. Results

3.1. Depressive Behavior

Intrastriatal injection of 6-OHDA resulted in development of depressive behavior in mice. The consumption of sucrose, indicative of 6-OHDA-induced anhedonia, is presented in Figure 1A. In the three weeks after 6-OHDA treatment, sucrose consumption decreased significantly, reflecting progressive anhedonia. (Figure 1A; Control: 72.14 \pm 2.23%, n = 18; 6-OHDA: 55.05 \pm 3.43%, n = 18; p < 0.05). Glucose consumption was further decreased after seven weeks (Figure 1A; Control 72.79 \pm 1.33%, n = 6; 6-OHDA: 51.68 \pm 3.37%, n = 6; p < 0.05). Administration of fluoxetine and L-dopa for four weeks ameliorated 6-OHDA-induced decrease of sucrose consumption, albeit only partially (Flu: 76.05 \pm 4.55%, n = 6; 6-OHDA + Flu: 65.18 \pm 3.17%, n = 6; L-dopa: 75.43 \pm 2.74%, n = 6; 6-OHDA + L-dopa: 63.03 \pm 3.95%, n = 6; p < 0.05).



Figure 1. Effects of L-dopa and fluoxetine on depressive behavior (sucrose preference test, tail suspension test, and forced swimming test) induced by 6-Hydroxydopamine (6-OHDA). After three weeks recovery from the 6-OHDA lesions, mice with anhedonia were daily injected intraperitoneally with fluoxetine (10 mg/kg/d) or L-dopa (20 mg/kg/d in saline and combined with 12 mg/kg of benserazide) for four weeks. Behavior tests were performed just before or after L-dopa or fluoxetine treatment. (A) Percentage of sucrose preference in sucrose preference test. Values were expressed as the mean \pm standard error of the mean (SEM). * Indicates statistically significant (p < 0.05) difference from other groups at the same treatment period. ** Indicates statistically significant (p < 0.05) difference or L-dopa. (B) The duration of immobility in tail suspension test. Values are expressed as the mean \pm SEM. * Indicates statistically significant (p < 0.05) difference from other groups at the same treatment period. ** Values are expressed as the mean \pm SEM. * Indicates statistically significant (p < 0.05) difference from other groups at the same treatment (p < 0.05) difference from other groups at the same treatment period. ** Indicates are expressed as the mean \pm SEM. * Indicates statistically significant (p < 0.05) difference from other groups at the same treatment period. (C) The duration of immobility in forced swimming test. Values are expressed as the mean \pm SEM. * Indicates statistically significant (p < 0.05) difference from other groups at the same treatment period.

The duration of immobility of tail suspension test is presented in Figure 1B. Three weeks after 6-OHDA treatment, the duration of immobility increased significantly (Control: 165.14 ± 10.01 s, n = 18; 6-OHDA: 219.29 ± 7.70 s, n = 18; p < 0.05) and it was further increased after seven weeks (Control: $173.75 \pm 5.45\%$, n = 6; 6-OHDA: $240.00 \pm 8.80\%$, n = 6; p < 0.05). Administration of fluoxetine and L-dopa for four weeks removed 6-OHDA-induced increase of duration of immobility (Flu: 180.40 ± 10.87 s, n = 6; 6-OHDA + Flu: 180.20 ± 9.30 s, n = 6; L-dopa: 177.60 ± 11.76 s, n = 6; 6-OHDA + L-dopa: 206.33 ± 10.59 s, n = 6; p < 0.05).

In the forced-swimming test (Figure 1C), injection of 6-OHDA significantly increased the time of immobility at three weeks after surgery (Control: 55.08 ± 4.55 s, n = 18; 6-OHDA: 85.16 ± 6.85 s, n = 18; p < 0.05). Immobility time was further increased after seven weeks (Control: 54.75 ± 2.84 s, n = 6; 6-OHDA: 95.40 ± 8.41 s, n = 6; p < 0.05). Administration of fluoxetine and L-dopa for four weeks

corrected 6-OHDA-induced increase of time of immobility (Flu: 52.00 ± 9.99 s, n = 6; 6-OHDA + Flu: 67.80 ± 7.59 s, n = 6; L-dopa: 52.80 ± 5.23 s, n = 6; 6-OHDA + L-dopa: 65.67 ± 8.71 s, n = 6; p < 0.05).

The results of time spent in the central area in the open field test are presented in Figure 2A. Three and seven weeks after exposure to 6-OHDA, there was no change in time spend in the central in open field. However, the drug decreased the number of squares crossed (three weeks: Control: 85.12 ± 15.27 , n = 18; 6-OHDA: 51.53 ± 5.44 , n = 18; p < 0.05; seven weeks: Control: 46.60 ± 5.96 , n = 6; 6-OHDA: 27.00 ± 3.69 , n = 6; p < 0.05) and frequency of rearing (three weeks: Control: 15.11 ± 2.24 , n = 185; 6-OHDA: 10.79 ± 1.44 , n = 18; p < 0.05; seven weeks: Control: 14.40 ± 1.86 , n = 6; 6-OHDA: 8.20 ± 0.97 , n = 6; p < 0.05) after three and seven weeks of treatment (Figure 2B,C). Administration of fluoxetine and L-dopa for four weeks corrected 6-OHDA-induced decrease of square crossed (Flu: 46.00 ± 4.46 , n = 6; 6-OHDA + Flu: 45.00 ± 6.27 , n = 6; L-dopa: 44.00 ± 3.22 , n = 6; 6-OHDA + L-dopa: 44.80 ± 8.41 , n = 6; p < 0.05) and frequency of rearing (Flu: 14.40 ± 1.17 , n = 6; 6-OHDA + Flu: 15.00 ± 2.24 , n = 6; L-dopa: 13.80 ± 1.85 , n = 6; 6-OHDA + L-dopa: 15.80 ± 2.71 , n = 6; p < 0.05).



Figure 2. Effects of L-dopa and fluoxetine on open field test in mice treated with 6-OHDA. After three-weeks recovery from the 6-OHDA lesions, mice with anhedonia were daily injected intraperitoneally with fluoxetine (10 mg/kg/d) or L-dopa (20 mg/kg/d in saline and combined with 12 mg/kg of benserazide) for four weeks. Behavior tests were performed just before or after L-dopa or fluoxetine treatment. (**A**) The time spent in the central square in the open field test. The values are expressed as the mean \pm SEM. (**B**) The number of squares crossed in open field test. The values are expressed as the mean \pm SEM. * Indicates statistically significant (p < 0.05) difference from other groups at the same treatment period. (**C**) Frequency of rearing in open field test. The values are expressed as the mean \pm SEM. * Indicates statistically significant (p < 0.05) difference from other groups at the same treatment period.

3.2. Motor Activity

In the rotarod performance test, the latency of fall (Figure 3A) in mice treated with 6-OHDA was significantly shorter than in control groups after three weeks (Control: 69.30 ± 4.27 s, n = 18; 6-OHDA: 57.13 ± 4.58 s, n = 18; p < 0.05) and after seven weeks (Control: 53.10 ± 6.26 s, n = 6; 6-OHDA: 31.90 ± 2.04 s, n = 6; p < 0.05) of 6-OHDA treatment. Although L-dopa corrected 6-OHDA-induced decrease of latency of fall after seven weeks, fluoxetine had no effect (Flu: 49.10 ± 6.19 s, n = 6; 6-OHDA + Flu: 29.20 ± 5.84 s, n = 6; L-dopa: 50.70 ± 8.74 s, n = 6; 6-OHDA + L-dopa: 49.90 ± 9.43 s, n = 6; p < 0.05).



Figure 3. Effects of L-dopa and fluoxetine on motor activity (the rotarod test and the pole test) in mice treated with 6-OHDA. After three-weeks recovery from the 6-OHDA lesions, mice with anhedonia were daily injected intraperitoneally with fluoxetine (10 mg/kg/d) or L-dopa (20 mg/kg/d in saline and combined with 12 mg/kg/d of benserazide) for four weeks. Behavior tests were performed just before or after L-dopa or fluoxetine treatment. (A) The latency to fall off the rotarod in the rotarod test. The values are expressed as the mean \pm SEM. * Indicates statistically significant (p < 0.05) difference from other groups at the same treatment period. (B) T-turn time in the pole test. The values are expressed as the mean \pm SEM. * Indicates statistically significant (p < 0.05) difference from other groups at the same treatment period. (B) T-turn time in the pole test. The values are expressed as the mean \pm SEM. * Indicates statistically significant (p < 0.05) difference from other groups at the same treatment period. (B) T-turn time in the pole test. The values are expressed as the mean \pm SEM. * Indicates statistically significant (p < 0.05) difference from other groups at the same treatment period.

The time taken by the mice to turn completely downward (Figure 3B; T-turn time) was not significantly affected by 6-OHDA after three weeks (Control: 2.15 ± 0.12 s, n = 18; 6-OHDA: 2.27 ± 0.30 s, n = 18; p > 0.05) and seven weeks (Control: 1.62 ± 0.32 s, n = 6; 6-OHDA: 1.80 ± 0.13 s, n = 6; p > 0.05). However, the time taken by the mice to reach the floor (Figure 3C; T-LA time) was

significantly increased after three (Control: 17.36 ± 1.32 s, n = 18; 6-OHDA: 23.92 ± 0.99 s, n = 18; p < 0.05) and seven weeks (Control: 15.75 ± 2.56 s, n = 6; 6-OHDA: 27.33 ± 1.36 s, n = 6; p < 0.05). Again, L-dopa corrected 6-OHDA-induced increase of T-turn time after seven weeks, but fluoxetine had no effect (Flu: 16.50 ± 3.13 s, n = 6; 6-OHDA + Flu: 26.70 ± 1.76 s, n = 6; L-dopa: 16.80 ± 2.44 s, n = 6; 6-OHDA + L-dopa: 18.90 ± 2.83 s, n = 6; p < 0.05).

3.3. Expression of mRNA and Protein of 5-HT_{2B} Receptor

In cerebral tissue of mice treated with 6-OHDA, the mRNA level of 5-HT_{2B} receptor decreased to $43.8 \pm 4.7\%$ of control groups (n = 3, p < 0.05) and protein expression to $67.3 \pm 4.3\%$ (n = 3, p < 0.05) seven weeks after lesion (Figure 4A,B). Experiments with freshly isolated astrocytes and neurons from transgenic mice demonstrated that the decrease of 5-HT_{2B} receptor mRNA expression in the in vivo brain was confined to astrocytes and was not detected in neurons (Figure 4C). However, this downregulation was corrected by L-dopa (Figure 4A,B) or fluoxetine (Figure 4D,E) that was injected three weeks after 6-OHDA treatment and continued for four weeks.



Figure 4. Effects of L-dopa and fluoxetine on expression of mRNA and protein of 5-HT_{2B} receptor in brains of anhedonia mice induced by 6-OHDA. After three-weeks recovery from the 6-OHDA lesions, mice with anhedonia were daily injected intraperitoneally with fluoxetine (10 mg/kg/d) or L-dopa (20 mg/kg/d in saline and combined with 12 mg/kg/d of benserazide) for four weeks.

In some experiments, (C) freshly isolated astrocytes and neurons from transgenic mice were used for determination of 5-HT_{2B} receptor mRNA expression. (A,C,D) mRNA expression measured by reverse transcription-polymerase chain reaction (RT-PCR) of (A) 5-HT_{2B} receptor in cerebral cortex in vivo from anhedonia mice treated with L-dopa for four weeks, (C) in freshly isolated astrocytes and neurons from transgenic mice, or (D) in cerebral cortex in vivo from anhedonia mice treated with fluoxetine for four weeks. A representative experiment showing mRNA for 5-HT_{2B} receptor and for TATA box-binding protein (TBP), as a housekeeping gene. The size of PCR product of 5-HT_{2B} receptor is 370 bp and that of TBP 236 bp. Similar results were obtained in three independent experiments. Average mRNA expression was quantified as the ratio between 5-HT_{2B} receptor and the housekeeping TBP gene. Ratios between 5-HT_{2B} receptor and TBP in control group were designated a value of one. Standard error of the mean (SEM) values are indicated by vertical bars. * Indicates statistically significant (p < 0.05) difference from all other groups. (**B**,**E**) Protein expression measured by immunoblotting of 5-HT_{2B} receptor in cerebral cortex in vivo from anhedonia mice treated with (B) L-dopa or fluoxetine (E) for four weeks. Immunoblots from representative experiments. Bands of 55 kDa and 42 kDa represent 5-HT_{2B} receptor (5-HT_{2B}R) and β -actin, respectively. Similar results were obtained in three independent experiments. Average protein level was quantified as ratios between 5-HT_{2B}R and β -actin. Ratios between 5-HT_{2B} receptor and β -actin in control group were designated a value of one. Standard error of the mean values are indicated by vertical bars. * Indicates statistically significant (p < 0.05) difference from all other groups.

The time course of expression of 5-HT_{2B} receptor mRNA during 6-OHDA treatment in cultured astrocytes is shown on Figure 5A. At 6 h and 12 h, there was no difference in 5-HT_{2B} receptor expression in control and 6-OHDA groups. However, after 24 h treatment, mRNA of 5-HT_{2B} receptor decreased to 64.7 \pm 6.2% of control groups (n = 3, p < 0.05). It was further decreased to 57.1 \pm 9.6% after 48 h of treatment (n = 3, p < 0.05). Expression of 5-HT_{2B} receptor protein showed similar pattern as mRNA (Figure 5B). After 24 h-exposure to 6-OHDA, cultures were treated with L-dopa or fluoxetine for another two weeks. Both L-dopa at 10 μ M and fluoxetine at 1 μ M abolished effect of 6-OHDA (Figure 5C–F).



Figure 5. Effects of L-dopa and fluoxetine on 6-OHDA induced downregulation of expression of mRNA and protein of 5-HT_{2B} receptor in primary cultures of astrocytes. (**A**) Effects of 6-OHDA on mRNA and protein expression of 5-HT_{2B} receptor in primary cultures of astrocytes. Cells were treated with saline (Control) or 6-OHDA (50 μ M) for 6 h, 12 h, 24 h and 48 h. (**B**) Effects of L-dopa on 6-OHDA induced downregulation of expression of mRNA and protein of 5-HT_{2B} receptor in primary cultures of astrocytes.

Cells were treated with L-dopa (10 μ M) for two weeks after 24 h 6-OHDA exposure. (C) Effects of fluoxetine on 6-OHDA induced downregulation of expression of mRNA and protein of 5-HT_{2B} receptor in primary cultures of astrocytes. Cells were treated with fluoxetine (1 μ M) for two weeks after 24 h 6-OHDA exposure. Representative experiments show mRNA expression for 5-HT_{2B} receptor and for TBP, as a housekeeping gene (top panels). The size of PCR product of 5-HT_{2B} receptor is 370 bp and that of TBP 236 bp. Similar results were obtained in three independent experiments. Average mRNA expression was quantified as ratio between 5-HT_{2B} receptor and the housekeeping TBP gene. Ratios between 5-HT_{2B} receptor and TBP in control group were designated a value of one. Immunoblots show bands of 55 kDa and 42 kDa representing 5-HT_{2B} receptor and β -actin, respectively. Similar results were obtained in three independent experiments aratios between 5-HT_{2B}R and β -actin. Ratios between 5-HT_{2B} receptor and β -actin in the control group were designated a value of one. Standard error of the mean values are indicated by vertical bars. * Indicates statistically significant (*p* < 0.05) difference from other groups at the same treatment period.

4. Discussion

Astrocytes, being intimately associated with synaptic structures, regulate neurotransmission, synaptic plasticity, and integration in neuronal networks through multiple mechanisms that control synaptogenesis, maintain ion and neurotransmitter homeostasis in the synaptic cleft, provide neuronal terminals with neurotransmitter precursors, and contribute to synaptic extinction [25–29]. Pathological changes to astroglia, therefore, may significantly affect brain function and lead to neurological disorders [30]. In particular, astrocytopathies, mainly in the form of atrophy, decrease in astroglial densities, and possibly loss in astroglia homeostatic function, contribute to major neuropsychiatric diseases such as major depression and schizophrenia [8,9,31,32]. Expression of astroglial serotonin receptors, which mediate neuronal–glial interactions, are modified in major depression and bipolar disorders; and antidepressants correct these pathological changes [33,34].

Previously, we reported that the decrease of gene expression of astrocytic 5-HT_{2B} receptor parallels the development of depressive behavior in the MPTP mouse model of PD, while fluoxetine ameliorates both the decrease in 5-HT_{2B} receptor expression and anhedonia [13]. In this study, we describe similar results obtained in 6-OHDA mouse model of PD. These new findings further corroborate the notion that the decrease in gene expression of astroglial 5-HT_{2B} receptors may be associated with pathophysiological evolution of PD-associated depression.

The MPTP is a lipidophilic compound able to penetrate the blood-brain barrier (BBB). Consequently, MPTP can be injected systemically to induce a bilateral parkinsonism, or, when infused through the carotid artery, to induce hemiparkinsonism [35]. In the latter settings, contralateral hemisphere can be used as control [36]. After crossing the BBB, MPTP (which by itself is non-toxic) is accumulated in astrocytes, where monoaminoxidase-B converts it to the toxic metabolite, MPP+. The latter is released from astrocytes and is accumulated by dopaminergic neurons through the dopamine transporter. In neurons, the MPP+ inhibits mitochondrial complex I, thus affecting adenosine triphosphate (ATP) synthesis, boosting production of reactive oxygen species (ROS), and leading to cell death [37]. After intraperitoneal injection (which we used in a previous study), MPTP spreads throughout the brain. Astrocytes thus may be affected directly by the drug as we have seen in cultured astrocytes [13], irrespective to the deficiency of dopaminergic system. In this study, we used 6-OHDA, a compound that cannot cross the BBB. 6-Hydroxydopamine enters dopaminergic neurons by dopamine transporter and thereafter triggers the production of neurotoxic ROS [36]. In contrast to MPTP, which is converted in neurotoxic agent by astrocytes, 6-OHDA enters neurons causing their demise. The usage of the 6-OHDA model therefore excludes possible direct damage to astrocytes which might be present in MPTP-treated animals. 6-Hydroxydopamine induces nigral dopamine cell loss and dopamine depletion. However, it does not seem to affect other brain regions, such as olfactory structures, lower brain stem areas, or locus coeruleus [14]. Therefore, the decrease of 5-HT_{2B} receptor in the brain in vivo may be related to the aberrations in serotonergic, adrenergic, or dopaminergic neurotransmission [38], although we cannot exclude the possibility of direct drug

effect on astrocytes in cerebral hemispheres since 6-OHDA also decreases gene expression of $5-HT_{2B}$ receptor in cultured astrocytes.

L-Dopa is a precursor of dopamine; L-dopa crosses the BBB and is converted to dopamine by aromatic amino acid decarboxylase [39]. In the clinical treatment of PD, L-dopa is used to replenish dopamine pool and is therapeutically effective in both PD patients and animal models of the disease [40]. However, it is controversial whether L-dopa has effect on PD depression. The clinical data show that L-dopa has no effect or accelerates depression or anxiety (see [4] and references therein). Similar results were obtained from animal models of PD [4]. Our data show that treatment with L-dopa for four weeks ameliorates both 6-OHDA-induced decrease of 5-HT_{2B} receptor expression in the brain in vivo and 6-OHDA-induced depressive behavior, suggesting the link between 5-HT_{2B} receptors and 6-OHDA-induced depression. Astrocytes express neutral amino acid transporter (LAT/SLC7A5) and dopamine transporter (DAT/SLC6A3) [41–45]; astroglia mainly function as a reservoir of L-dopa that regulates the uptake or release of L-dopa depending on extracellular L-dopa concentration, but are less capable of converting L-dopa to dopamine [46]. The effect L-dopa on ROS production is debatable. When L-dopa is decarboxylated to dopamine (DA) by aromatic L-amino acid decarboxylase (AADC), ROS are generated that could ultimately lead to cell death [47]. Nevertheless, L-dopa in some in vivo and in vitro experiments had no toxic effects, or even showed antioxidant capabilities [48–51]. Oxidative stress in peripheral blood mononuclear cells from patients with PD is negatively correlated with L-dopa dosage [48]. Experiments with catecholaminergic human neuroblastoma cells showed that L-dopa may have a protective effect on dopaminergic cells [49]. Similar results were also obtained in PC12 cells [52]. In the perinatal 6-OHDA lifelong model of PD, elevated basal levels of ROS occurring in denervated dopaminergic striatum are suppressed by L-dopa treatment [50]. L-Dopa in follicular fluid is an antioxidant factor and exerts positive influences on cultured human granulosa cells, whereas DA derived from L-dopa has opposite actions [51]. In the present work, we have found that L-dopa corrects 6-OHDA-induced decrease of 5-HT_{2B} receptor in astrocytes in primary cultures, suggesting L-dopa that may protect cells by its antioxidant effects.

In contrast to L-dopa, fluoxetine has no effect on 6-OHDA-induced motor deficiency, suggesting the effect of L-dopa on motor activity is dependent on DA replenishment. However, the effect of fluoxetine on depressive behavior develops in parallel with its effect on the gene expression of the 5-HT_{2B} receptor [10]. The effects of fluoxetine on astrocytes are mediated by the 5-HT_{2B} receptor. The affinity 5-HT to the astroglial 5-HT_{2B} receptor is substantially higher than to 5-HT_{2C} receptor [53]. Different SSRIs bind to and activate astroglial 5-HT_{2B} receptors, which induce EGFR transactivation [54]. Stimulation of EGFR-dependent signaling cascades regulates expression of multiple genes (for a review, see [55,56]). In mice that develop anhedonia following chronic stress, expression of astroglial 5-HT_{2B} receptor is significantly suppressed; at the same time, expression of 5-HT_{2B} receptor does not change in mice which do not develop anhedonia [11]. Chronic treatment with fluoxetine rescues this deficit and increases expression of 5-HT_{2B} receptors in astrocytes in the brains of anhedonic animals [12]. Similarly, astrocytic 5-HT_{2B} receptor is downregulated only in anhedonic mice, but not in those that do not develop anhedonia in MPTP-induced PD model animals. This is in agreement with our present findings that astrocytic 5-HT_{2B} receptor is decreased in depressed animals in 6-OHDA PD model.

Pathophysiology of depression associated with PD is not clear. We have shown that downregulation of the gene expression of 5-HT_{2B} receptors occurs specifically in astrocytes in parallel with the development of depressive behavior in both 6-OHDA and MPTP animal models of PD. The relevance of 5-HT_{2B} receptors to depression is corroborated by (1) the decrease of its gene expression in the nervous tissue of animals developing depressive behavior under the CMS [11,57], in the MPTP PD animal model [13], and in the 6-OHDA PD animal model; (2) the upregulation of 5-HT_{2B} receptor gene expression by chronic treatment with fluoxetine in astrocytes in cultures and freshly isolated from the in vivo brains [11,57]; and (3) the dependence of antidepressant effect of fluoxetine on 5-HT_{2B} receptors in vivo [58]. Since both drugs directly decrease expression of 5-HT_{2B}

receptors in cultured astrocytes, there is still a possibility that this phenomenon may only occur in PD animal models. To make a conclusion, postmortem examination of 5-HT_{2B} receptors in PD patients' brains is needed.

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