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Effects of repeated 5-HT_6 receptor stimulation on BDNF gene expression and cell survival

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HIGHLIGHTS

- We have previously shown an acute onset by the selective 5-HT_6 agonist LY-586713 on hippocampal *bdnf* expression.
- Here we show that this effect was still present following sub-chronic (4 days), but not chronic (14 days), treatment.
- The effect on 5-HT_6-mediated cell survival was also dependent on a similar length of treatment.
- Taken together this suggests a time dependent desensitization of a potential neurotrophic action by 5-HT_6 receptors.

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ABSTRACT

In support of the neurotrophic hypothesis of depression chronic antidepressant drug treatment increases brain-derived neurotrophic factor (*bdnf*) gene expression and neurogenesis. Regarding 5-HT active drugs, the 5-HT receptor behind these effects remains unidentified. Here we report the effect of repeated 5-HT_6-receptor stimulation on *bdnf* expression and cell survival. The previously reported acute stimulatory action of the selective 5-HT_6 agonist LY-586713 on hippocampal *bdnf* expression was still present following sub-chronic (4 days), but not chronic (14 days), treatment. The effect on 5-HT_6-mediated cell survival was also dependent on a similar length of treatment. Hence, our study found no support for a primary effect of 5-HT_6 receptors in the mediation of chronic antidepressant drug-induced up-regulation of *bdnf* expression or neurogenesis.

1. Introduction

It is now over a decade ago since it was first shown that chronic but not acute antidepressant drug treatment increases brain-derived neurotrophic factor (BDNF) mRNA levels in rat brain regions, including the hippocampus. These initial *bdnf* expression studies formed the basis for the "neurotrophin hypothesis of depression", which postulates that central BDNF deficiencies underlie depression, and that antidepressants work via the restoration of central BDNF activity [7,14,15]. Clinical support for the neurotrophin hypothesis includes studies demonstrating low hippocampal and serum BDNF levels in un-medicated depressed patients, and that these levels increase with antidepressant drug treatment [3,8,25]. The effects of antidepressant drugs on *bdnf* expression through the blockade of the serotonin transporter are however complex and depend strictly on the time after the last injection as well as treatment duration. Thus, while acute (single) and sub-chronic (4 days) of administration fail to increase *bdnf* expression, chronic (2–3 weeks) causes an up-regulation of *bdnf* [4,5,9]. In spite of a plausible role for an interaction between BDNF and 5-HT in the treatment of depression, surprisingly little is known regarding individual 5-HT receptor subtypes and their actions on *bdnf* expression and neurogenesis. However, preclinical data suggest a possible role for 5-HT_6 receptors in depression and its treatment [2]. This includes previous biochemical and behavioural evidence for antidepressant effects of acute 5-HT_6-receptor stimulation [16,23]. In support of this we have previously shown that a single administration of the selective 5-HT_6 receptor agonist LY-586713 enhances *bdnf* expression, making this receptor subtype a possible candidate for also mediating the increase in *bdnf* expression following chronic treatment with antidepressant drugs enhancing extracellular 5-HT levels including fluoxetine [6].

In the present study, we investigated if the previously reported acute stimulatory action of the selective 5-HT_6 agonist LY-586713 on hippocampal *bdnf* expression prevails following sub-chronic (4 days) and chronic (14 days) treatment. We also examined if the

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5-HT_6 agonist resulted in hippocampal neurogenesis (i.e., cell proliferation and survival), an effect shown to be influenced by BDNF and required for the behavioural effects of antidepressants [20,21].

2. Materials and methods

2.1. Animals

All procedures were carried out in accordance with the UK Animal Scientific Procedure Act (1986). Male Sprague-Dawley rats (225–250 g, Charles River, UK) were housed six per cage and allowed at least 10 days of habituation in the animal facilities before the start of any experimental procedure. Animals were kept under a 12-h light/dark cycle in a temperature-controlled environment, with free access to food and water. All efforts were made to minimize animal suffering, reduce the number of animals used and to utilize alternatives to in vivo techniques, if available.

2.2. Drug treatment protocols

For chronic (14 days of treatment) or sub-chronic (4 days of treatment) groups of six rats were treated once daily (9 am) with LY-586713 (1 mg/kg, s.c.) or the corresponding vehicle (25% (w/v) hydroxypropyl β-cyclodextrin in saline, 1 ml/kg, i.p.). Dose, injection route and time after treatment were chosen according to our previous study, which demonstrated acute pharmacological activity by LY-586713 on brain *bdnf* expression, an effect completely blocked by the selective 5-HT_6-antagonist SB-271046 [6]. In all treatment groups, rats were sacrificed at 18 h after the last injection.

2.3. In situ hybridization procedure

Brain sections (10 μm) were cut on a cryostat, thaw-mounted onto gelatin-subbed slides and pre-treated for in situ hybridization, using a standard protocol described previously [18]. An oligonucleotide DNA probe complimentary to all *bdnf* transcripts (exon IX) [1] (5′-GGT CTC GTA GAA ATA TTG CTT CAG TTG GCC TTT TGA-3′) was identified using a basic BLAST search (National Institute for Health website: www.ncbi.nlm.nih.gov) and customer synthesized (Eurogentec DNA Service Ltd, Southampton, UK).

The oligonucleotide was 3′-tail labelled with ^{35}S-dATP with terminal deoxynucleotide transferase. The labelled oligonucleotide probe (specific activity >10^9 cpm/μg) was added to each section (1×10^6 cpm/section) in hybridization buffer, as previously described [28]. Incubation was conducted in humidity chambers (containing 50% formamide in 4× SSC) at calculated incubation temperature of 29.3 °C for 14–16 h. Slides were then washed in 1× SSC buffer at 52 °C for 3× 20 min, followed by 2× 60 min at room temperature. Sections were air-dried and exposed to autoradiography film (Biomax, Amersham, UK) for 5–7 days at room temperature. Controls included the use of oligonucleotides in sense orientation and displacement with unlabelled probes. Searches conducted with the GenBank database using the BLAST program revealed no significant homology of the nucleotide sequences with other previously characterized rat brain genes.

2.4. BrdU labelling and immunohistochemistry protocols

Groups ($n = 6$) of rats previously administered for 4 days (sub-chronic) or 14 days (chronic) with daily LY-586713 injections (1 mg/kg, s.c.) were injected with 4× BrdU (75 mg/kg in PBS, i.p.) every 2 h, 24 h after the last LY-586713 administration. For measurements of cell proliferation and cell survival, rats were killed 24 h and 28 days after the last BrdU injection respectively. Rats were transcardially perfused with saline, followed by 4% ice-cold paraformaldehyde (PFA). Brains were removed, post-fixed in 4% PFA overnight, placed in 30% sucrose in phosphate buffered saline (PBS) for 3 days, removed from sucrose and frozen at −70 °C. Coronal sections (40 μm) through the hippocampus (−2.40 mm to −4.80 mm, relative to bregma) [17], were cut on a cryostat and stored in a cryoprotectant solution at −20 °C prior to use in the immunocytochemistry procedure. Following rinsing in PBS, sections were incubated in 1% H_2O_2 in PBS for 10 min, followed by 3 × 10 min PBS washes.

2.5. Quantification of BrdU-labelling

Counting methods for BrdU-positive cells were adapted from previous studies [10,26]. Every sixth hippocampal section (240 μm apart) from each animal was processed for BrdU-labelling. This was done to ensure that the same neuron was not counted in more than one section. Using a light microscope set at ×40 objective, BrdU-positive cells were counted, blind to the treatment group, by drawing each cell onto a paper drawing corresponding to the region of the dentate gyrus being analysed. Labelled cells included in the count were those that were in or touching the subgranular zone (SGZ) and granular layer of the dentate gyrus (GCL) and those within the dentate hilus, which was taken as the complete area between the opposite arms of the dentate gyrus SGZ/GCL (Fig. 3). All cells were counted regardless of size or shape. The focus of the microscope was adjusted by hand to count BrdU-labelled cells in different focal planes.

2.6. Data collection and analysis

The relative abundance of total *bdnf* was determined by densitometric quantification of autoradiograms with correction for non-specific signals. Developed films were digitized and analysed using a computerized image-capture and analysis system (MCID-4, Imaging Research, St. Catherine's, Ontario, Canada). Optical density values were converted to nCi/g tissue using a standard curve generated with ^{14}C standards, calibrated against ^{35}S standards to correct for non-linearity. Three sections from each brain hybridized with the radioactive probe were measured and the mean of the values from these three sections used to quantify mRNA levels for the selected brain region from each animal. Graphing was performed using Excel (Microsoft Corporation). Statistical analysis of data was performed using SPSS for Windows v.10.0 (SPPS Inc., Chicago, USA). Comparisons between two groups (vehicle control and drug-treated) were made using Student's unpaired two-tailed *t*-test. When appropriate multiple comparisons were determined by two-way ANOVA followed by Bonferroni post hoc analysis. Significance was determined at $P < 0.05$.

3. Results

3.1. Effect of repeated administration with the selective 5-HT_6 agonist LY-586713 on hippocampal bdnf levels

We have previously shown that a single administration of the selective 5-HT_6 agonist LY-586713 (1 mg/kg, s.c.) increases *bdnf* expression in regions of the hippocampus at 24 h [6]. Here, we investigated the action of LY-586713 at the same dose after sub-chronic (once daily for 4 days) and chronic (14 days) administration on *bdnf* expression. Four days of LY-586713 administration up-regulated *bdnf* expression in hippocampus. This effect reached significance in the CA1 ($P < 0.01$) and CA3 ($P < 0.05$), but not in the DG, compared to vehicle controls. In contrast, chronic treatment for 14 days resulted in no significant change in *bdnf* expression in any of the hippocampal brain region analysed. Two-way ANOVA