The role of 5-HT receptor subtypes in the regulation of brain derived neurotrophic factor (BDNF) gene expression

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Abstract

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 the rat hippocampus. These initial *bdnf* expression studies were soon followed by studies showing that the effects by antidepressant drugs on *bdnf* expression are more complex than originally stated. Thus, the selective serotonin re-uptake inhibitors (SSRIs) produce a 'biphasic effect' on *bdnf* levels, involving a down-regulation at 4 hours (both acute and chronic treatment) and an increase at 24 hours (only after chronic treatment). Surprisingly few studies have attempted to determine the role of 5-HT and its different receptors in the mediation of these effects. Therefore, the present study aimed to investigate the role of 5-HT receptor subtypes in mediating the two opposing effects of the SSRI fluoxetine on hippocampal *bdnf* expression. Our study shows that the transient fluoxetine-induced down-regulation of the *bdnf* gene expression depends on an intact 5-HT, but not noradrenaline, system or circulating glucocorticoids. Pre-treatment with the 5-HT_4 antagonist SB 204070 blocked the overall fluoxetine-induced inhibition of *bdnf* levels in hippocampus, while pre-treatment with the 5-HT_2 antagonists ketanserin had an effect in the CA3 but not in the dentate gyrus. However, the 5-HT_{1A} antagonist WAY100,635 and the 5-HT_3 antagonist granisetron were both ineffective. In comparison, the previously reported acute (single administration) 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 little support for a primary effect of 5-HT_6 receptors in the mediation of chronic fluoxetine-induced up-regulation of *bdnf* expression or neurogenesis.

1. Introduction

It is now over a decade ago since it was first shown that chronic 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 (Duman et al., 1997; Neto et al., 2011). Clinical support for the neurotrophin hypothesis has been shown in studies demonstrating low hippocampal and serum BDNF levels in un-medicated depressed patients, and that these levels increase with antidepressant drug treatment (Chen et al., 2001; Gorgulu and Caliyurt, 2009; Wolkowitz et al., 2011). Not all studies, however, fully support the neurotrophin hypothesis of depression (Groves, 2007) and recent findings point more towards BDNF involvement in the treatment than in the cause of depression (Martinowich et al., 2007). In addition, the effects of antidepressant drugs on *bdnf* expression through the blockade of serotonin transporter (i.e., SSRIs) are complex and depend on both treatment duration and the time after the last injection (Coppell et al., 2003; De Foubert et al., 2004; Khundakar and Zetterstrom, 2006). Thus, chronic administration (2 weeks' treatment) of SSRIs, including fluoxetine, produced a 'biphasic effect' on *bdnf* levels, involving a down regulation at 4 hours and an up-regulation at 24 hours. While a single administration does not increase hippocampal *bdnf* at 24 hours, the inhibitory action at 4 hours is, however, still present (Coppell et al., 2003; Khundakar and Zetterstrom, 2006). Similarly, antidepressant drugs show a time-dependent biphasic modulation of human *bdnf* expression (Donnici et al., 2008). In spite of a plausible role for an interaction between BDNF and 5-HT in the treatment of depression, surprisingly little is known

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regarding individual 5-HT receptor subtypes and their actions on *bdnf* expression. In this respect, we have recently shown that the inhibitory action by the SSRI paroxetine on *bdnf* expression appears to be mediated indirectly via $GABA_B$ receptor stimulation (Khundakar and Zetterstrom, 2011). However, the 5-HT receptor subtype that initiates this effect (by possibly increasing GABA release) is not known. In addition, 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 SSRI treatment (de Foubert et al., 2007).

In the present study, we firstly sought to identify the 5-HT receptor subtype responsible for SSRI induced down-regulation of hippocampal *bdnf* levels previously detected at 4 hours after both acute and chronic treatment (Coppell et al., 2003) Secondly, 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. Finally, we examined if 5-HT_6 agonist administration resulted in hippocampal neurogenesis (i.e., cell proliferation and survival), an effect previously shown to be influenced by BDNF and required for the behavioral effects of antidepressants and (Sairanen et al., 2005; Santarelli et al., 2003).

2. Experimental procedures

2.1 Animals

All procedures were carried out in accordance with the UK Animal Scientific Procedure Act (1986). Normal male Sprague-Dawley rats (225–250 g, Charles River, UK) or adrenalectomized rats (225-250 g, Harlan-Olac, Bicester, UK) were housed four 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-hour 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

All drugs were dissolved in saline, with the exception for LY-586713, which was dissolved in 25% w/v hydroxypropyl ß-cyclodextrin (Sigma, UK) in saline. For acute treatment (single injections) animals were administered with fluoxetine (10 mg/kg, i.p.), renzapride (1 mg/kg, i.p.) or saline (1ml/kg, i.p.) and killed by decapitation 4 hours later. Separate groups of rats were pre-treated, 30 minutes before the second injection with the following selective 5-HT antagonist: The 5-HT_{1A} receptor antagonist N-[2-[4-(2-methoxyphenyl)-1-piperazineyl]ethyl]-N-(pyridinyl)-cyclohexanecarboximide-3-hydrochloride (WAY-100,635; 0.3mg/kg, s.c.), the 5-HT_2 receptor antagonist ketanserin (5mg/kg, i.p.), the 5-HT_3 receptor antagonist granisetron (1mg/kg i.p.) or the 5-HT_4 receptor antagonist 8-amino-7-chloro (N-butyl-4-piperidyl) methylbenzo-1,4-dioxan-5-carboxylate hydrochloride (SB-204070; 1mg/kg s.c.), or saline (1ml/kg, i.p.) prior to the injection of fluoxetine or renzapride (pre-treatment only with SB 204070) and sacrificed 4 hours after the last injection.

Doses and injection routes of all drugs were chosen according to previous literature, which have demonstrated robust pharmacological activity using *in viv*o models following injections (Barnes and Sharp, 1999; Coppell et al., 2003; De Foubert et al., 2004; de Foubert et al., 2007; Dumuis et al., 1989; Fletcher et al., 1996; Kennett et al., 1997; Nakayama, 2002). For chronic (14 days of treatment) or sub-chronic (4 days of treatment, only LY-586713) groups of rats were treated twice (9am and 5pm) daily with fluoxetine (10 mg/kg, i.p.), once daily with LY-586713 (1 mg/kg, s.c.) or the respective vehicles (1 ml/kg, i.p.) at the corresponding injection routes.

2.3. 5-HT and noradrenaline lesion protocols

In order to prevent 5,7-dihydroxytryptamine (5,7-DHT) from being taken up by noradrenergic neurons, rats were pre-treated with desipramine (25mg/kg, i.p.) 30 min prior to intracerebro-ventricular (i.c.v.) injection of 5,7-DHT or vehicle. For administration of 5,7-DHT (i.c.v.) or vehicle, halothane-anaesthetized rats were placed in a stereotaxic frame. The skull was exposed and a hole was drilled with stereotaxic guidance. The cannula was then slowly lowered into the right ventricle (AP 0.3, L 1.4, V 4.1 from bregma). 5,7-DHT (200 µg in 10 µl of vehicle) or vehicle (1% ascorbate-saline) was then infused over 5 minutes using a microinfusion pump (CMA). After the application of skin sutures, rats remained anaesthetized in the stereotaxic frame for up to 30 minutes before being returned to their home cages and allowed to recover for at least 2 weeks until further experimentation. To lesion central noradrenergic systems, rats were administered N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4; 50mg/kg i.p). The corresponding sham-lesioned control rats received 0.9% saline. Rats were recovered for one week before further experimentation. The extent of 5-HT and noradrenaline depletion following 5,7-DHT

and DSP-4 respectively was evaluated by analysis of hippocampal monoamine tissue content in the hippocampus from sham and lesioned rats using high-performance liquid chromatography with electrochemical detection (HPLC-EC). The regions of hippocampus were dissected out from individual rats, weighed and sonicated in, ice-cold 0.1M perchloric acid. The resulting suspension was then centrifuged for 5 min at 14 000 *g* at 4°C and the supernatant was assayed for content of 5-HT and noradrenaline using HPLC-EC (Zetterstrom et al., 1983).

2.4. 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 (Pei et al., 1997). An oligonucleotide DNA probe complimentary to all *bdnf* transcripts (exon IX) (Aid et al., 2007) (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 x 10^6 cpm/section) in hybridisation buffer, as previously described (Zetterstrom et al., 1998). Incubation was conducted in humidity chambers (containing 50% formamide in 4 x SSC) at calculated incubation temperature of 29.3 ^{o}C for 14–16 hours. Slides were then washed in 1 x SSC buffer at 52 ^{o}C for 3 x 20 minutes, followed by 2 x 60 minutes 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.

2.5. *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 x BrdU (75mg/kg in PBS, i.p.) every 2 hours, 24 hours after the last LY-586713 administration. For measurements of cell proliferation and cell survival, rats were killed 24 hours 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 PBS for 3 days, removed from sucrose and frozen at -70 ^{o}C. Coronal sections (40µm) through the hippocampus (-2.40 mm to -4.80 mm, relative to bregma, Paxinos and Watson, 1986), were cut on a cryostat and stored in a cryoprotectant solution at -20 ^{o}C prior to use in the immunocytochemistry procedure. Following rinsing in 0.02 M PBS, sections were incubated in 1% H_2O_2 in PBS for 10 min, followed by 3x10 min PBS washes.

2.6. Quantification of BrdU-labelling

Counting methods for BrdU-positive cells were adapted from previous studies (Malberg et al., 2000; Xu et al., 2004). 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 x 40 objective, BrdU-postive 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 4). 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. Taking into account that every sixth section was analysed, the total number of BrdU-labelled cells from each brain was multiplied by 6 to give the total number of BrdU-positive cells per dentate gyrus.

2.7. 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 were hybridized with probe 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. Multiple comparisons were determined using one-way ANOVA with post-hoc comparisons with Bonferroni post-hoc analysis. Significance was determined at $p<0.05$.

3. Results

3.1 Effects of 5-HT lesioning on acute fluoxetine-induced reduction of bdnf levels

Previous studies have shown that a single injection of a range of antidepressant drugs decrease hippocampal *bdnf* expression at 4 hours after the injection (Coppell et al., 2003; Khundakar and Zetterstrom, 2006; Khundakar and Zetterstrom, 2011). Here, we aimed to determine the involvement of 5-HT in this effect and therefore tested the effect of fluoxetine in 5-HT-lesioned rats. Intracerebroventricular injection of 5,7-DHT significantly ($p<0.01$) reduced hippocampal 5-HT levels by 85.3%; from 368±74 ng/g wet tissue in vehicle-injected controls (n=4) to 54±15ng/g in 5,7-DHT (200µg i.c.v, n=4) injected rats. Two weeks after injection, the 5,7-DHT did not significantly change basal hippocampal *bdnf* levels compared to sham treated rats. Fluoxetine significantly reduced *bdnf* levels in the dentate gyrus (-46%) and the CA1 (-26%), but not in CA3, in sham treated rats when compared to the corresponding saline injected sham controls. In contrast, in 5,7-DHT lesioned rats fluoxetine did not reduce *bdnf* levels, demonstrating the importance of an intact 5-HT system for this effect (Fig 1).

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3.2 Effects of noradrenaline lesioning on fluoxetine induced reduction of bdnf levels

Intraperitoneal injection of DSP-4 at one week significantly ($p<0.01$) reduced hippocampal noradrenaline levels by 73% from 817+107 ng/g wet tissue (saline injected controls, n=6) to 223+91 ng/g in rats injected with DSP-4 (50 mg/kg, n=6). The DSP-4 lesion per se did not alter *bdnf* levels in hippocampus compared to the corresponding sham group. Fluoxetine significantly decreased levels of *bdnf* in the

dentate gyrus of both sham (-25%) and DSP-4 (-19%) leisoned rats, but failed to show significant reductions in CA1 and CA3 for both treatment groups (Table 1).

3.3 Effects of circulating glucocorticoids on fluoxetine-induced reduction of bdnf levels

The effect of fluoxetine-induced down-regulation on hippocampal *bdnf* was examined in adrenalectomized rats. Adrenalectomy did not affect basal hippocampal *bdnf* expression. Neither did it prevent fluoxetine-induced inhibition of *bdnf* levels at 4 hours after a single injection. Thus, in adrenectomized rats (n=6), fluoxetine caused significant down regulation of *bdnf* in all three hippocampal subregions: dentate gyrus (-33%), CA1 (-18%) and in the CA3 (-17%) compared to the corresponding sham controls (n=6), (Table 2).

3.4 5-HT receptor subtypes and fluoxetine-induced inhibition of bdnf expression

In order to identify the role of different 5-HT receptor subtypes in the mediation of acute fluoxetine-induced down-regulation of hippocampal *bdnf,* rats were pre-treated with selective 5-HT antagonists 30 minutes before the injection of fluoxetine. When the selective 5-HT_4 antagonist SB-204070 (1 mg/kg, s.c.) was administered prior to fluoxetine (10 mg/kg, i.p.), there was a significant overall effect of the combined drug treatments on *bdnf* levels in dentate gyrus [$F(3,41)=13.585$, $P<0.001$], CA1 [$F(3,41)=8.320$, $P<0.001$] and CA3 [$F(3,41)=6.549$, $P<0.001$]. Post-hoc analysis demonstrated that fluoxetine at 4 hours decreased *bdnf* levels in all regions. This effect was completely blocked by pre-treatment with SB-204070, which had no effect when administered on its own (Fig 2a). Renzapride (1 mg/kg, i.p.), a full 5HT_4 agonist, reduced hippocampal *bdnf* expression. When SB-204070 (1 mg/kg, s.c.) was

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administered 30 minutes prior to renzapride, there was a significant overall effect of the combined drug treatments on *bdnf* levels in dentate gyrus [$F(3,16)=41.387$, $P<0.001$] and CA3 [$F(3,16)=9.700$, $P<0.001$] but not in CA1 [$F(3,16)=1.483$, $P=0.257$]. Post-hoc analysis demonstrated that renzapride decreased *bdnf* levels in dentate gyrus and CA3, but not in CA1, and these effects were completely blocked by pre-treatment with SB-204070 (fig 2b).

When the 5-HT_{1A} antagonist, WAY-100,635 (0.3mg/kg, s.c.) was administered prior to fluoxetine, there was a significant overall effect of the combined drug treatments on *bdnf* levels in dentate gyrus [$F(3,17)=21.078$, $P<0.001$], CA1 [$F(3,17)=19.044$, $P<0.001$] and CA3 [$F(3,17)=23.650$, $P<0.001$]. Post-hoc analysis demonstrated that fluoxetine decreased *bdnf* levels in all regions. This effect was unaltered by combined treatment with WAY-100,635, which had no effects on *bdnf* expression when administered on its own (Table 3).

When the 5-HT_2 antagonist ketanserin (5 mg/kg, i.p.) was administered prior to fluoxetine, there was a significant overall effect of the combined drug treatments on *bdnf* levels in dentate gyrus [$F(3,33)=21.840$, $P<0.001$], CA1 [$F(3,33)=3.225$, $P<0.035$] and CA3 [$F(3,33)=7.505$, $P<0.001$]. Post-hoc analysis demonstrated that fluoxetine decreased *bdnf* levels in dentate gyrus and CA3. Combined treatment with ketanserin did not alter the effect in dentate gyrus; whilst in the CA3 pre-treatment with the antagonist significantly attenuated the effect by fluoxetine. Ketanserin on its own had no effect on hippocampal *bdnf* expression (Table 3).

When the selective 5-HT_3 antagonist granisetron (1mg/kg, s.c.) was administered prior to fluoxetine there was a significant overall effect of the combined drug treatment on *bdnf* levels in dentate gyrus [$F(3,18)=21.344$, $P<0.001$] and CA3 [$F(3,18)=7.677$, $P<0.01$] but not CA1 [$F(3,18)=1.722$, $P=0.198$]. In this treatment

group, post-hoc analysis revealed that fluoxetine decreased *bdnf* levels in dentate gyrus but not CA1 or CA3 ($p=0.068$). These effects were unaltered by combined treatment with granisetron, which had no effect on *bdnf* expression on its own (Table 3).

3.5 Effects of 5-HT lesioning on chronic fluoxetine-induced increase of bdnf levels

As shown previously (Coppell et al., 2003; De Foubert et al., 2004; Khundakar and Zetterstrom, 2006; Nibuya et al., 1995; Russo-Neustadt et al., 2004), twice daily injections of fluoxetine for 14 days at 24 hours after the last injection significantly ($p<0.001$) increased *bdnf* levels in the dentate gyrus of the hippocampus (126$\pm$6.1, mean$\pm$s.e.m. values shown as % of controls, n=6) compared to the corresponding saline control group (100$\pm$2.8, n=6). In contrast, this effect failed to reach significance in the CA1 (109$\pm$3.4) and levels were even slightly reduced in the CA3 (93$\pm$3.1). In order to test the influence of 5-HT in mediating the fluoxetine-induced increase in the dentate gyrus, we investigated the effect in rats lesioned with 5,7-DHT 2 weeks before the start of the chronic fluoxetine treatment (i.e., twice daily injections for 14 days). Basal levels of *bdnf* expression at the end of the chronic treatment (i.e., 28 days after the 5,7-DHT lesion) were significantly ($p<0.05$) elevated in the dentate gyrus of lesioned rats (120$\pm$3.9%, n=6) compared to the corresponding sham controls (100$\pm$1.9%). In the CA1 and CA3, *bdnf* levels were, however, not different in 5,7-DHT lesioned rats compared to controls (data not shown). In contrast to the effect in non-lesioned rats, in animals lesioned with 5,7-DHT, 2 weeks prior to the start of the chronic drug schedule, fluoxetine failed to significantly increase *bdnf* levels in dentate gyrus (109$\pm$7.9%, n=6), compared to the corresponding 5,7-DHT-lesioned saline controls (100$\pm$3.9%, n=6).

3.6 Effect of repeated administration with the selective 5-HT$_6$ agonist LY-558713 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 hours (de Foubert et al., 2007). 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 analyzed (Figs 3a and 3b).

3.7 Effect of repeated administration with the selective 5-HT$_6$ agonist LY-558713 on cell proliferation and cell survival

The number of BrdU-positive cells was determined after twice-daily administration of LY-558713 for 4 and 14 days, with sacrifice 24 hours (cell proliferation) or 28 days (cell survival) after the last BrdU administration. As shown previously (Malberg et al., 2000; Nakagawa et al., 2002; Xu et al., 2004), BrdU-positive cells were mainly detected within the subgranular zone (SGZ) and the hilus of the dentate gyrus 24 hours after BrdU injections. At 28 days after the last BrdU injection, positive cells were also clearly visible in the granular cell layer (GCL) (Fig 4). The average total BrdU-positive cell counts for the whole dentate gyrus region measured from -2.40mm to -4.80, relative to Bregma (Paxinos and Watson, 1986), was approximately 2500 cells for the 24 hour treatment groups and 1500 cells for the 28 day treatment group,

corresponding with cell counts that that have been recorded in other published studies (Malberg et al., 2000; Nakagawa et al., 2002; Xu et al., 2004). The number of BrdU-labelled cells was always visible higher in the SGZ/GCL as compared to the hilus after all types of treatment. Neither, 4 or 14 days of LY-586713 had any significant effect on cell proliferation in the SGZ; while in the hilus, there was a small albeit significant reduction (-28±7.2 %, mean±S.E.M., n=6) at 4 days, but not at 14 days, compared to the corresponding vehicle treated control group. In comparison, 4 days of LY-586713 treatment significantly increased cell survival in both the SGZ/GCL (+26%) and the hilus (+36%) region of the dentate gyrus compared to the corresponding vehicle treated control group (Fig 5). In contrast this effect was not seen at 14 days (Fig 6).

4. Discussion

The SSRI type antidepressant drugs including fluoxetine produce a 'biphasic effect' on *bdnf* levels, involving a down-regulation at 4 hours (both acute and chronic treatment) and an increase at 24 hours (only after chronic treatment), (Coppell et al., 2003; Khundakar and Zetterstrom, 2006). The present study aimed to investigate the role 5-HT receptor subtypes in mediating these two opposing effects of SSRIs on hippocampal *bdnf* expression. Our study shows that the transient fluoxetine-induced down-regulation of *bdnf* expression depends on an intact 5-HT system and is blocked by the selective 5-HT_4 antagonist SB 204070. In comparison, the previously reported acute (single administration) 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 after chronic (14 days) treatment. The effect on 5-HT_6 mediated cell survival was also dependent on similar length of treatment.

In the present study, we confirm that after a few hours (i.e., 2-4 hours) of an acute administration fluoxetine decreases hippocampal levels of *bdnf* (Coppell et al., 2003; Dias et al., 2003; Khundakar and Zetterstrom, 2006; Miro et al., 2002), an effect also detected after 5-HT releasing drugs (Zetterstrom et al., 1999). The hippocampus receives serotonergic and noradrenergic innervation and microdialysis studies have shown that fluoxetine acutely increases hippocampal 5-HT and noradrenaline release (Gartside et al., 1995; Stanford, 1996). The present study therefore sought to investigate the role of 5-HT and noradrenaline in the fluoxetine-induced decrease of hippocampal *bdnf.* In 5,7-DHT-lesioned animals, with a reduction of hippocampal 5-HT levels by >85%, the fluoxetine-induced *bdnf* reduction was abolished. In rats pre-treated with the noradrenline-depleting agent DSP4, which caused a >70% reduction

of hippocampal noradrenaline, fluoxetine administration still resulted in significantly reduced *bdnf* expression, indicating a pivotal role for 5-HT but not noradrenaline in mediating this effect.

Glucocorticoids down regulate *bdnf* in the hippocampus (Barbany and Persson, 1992; Schaaf et al., 1999) and 5-HT activates the HPA axis (predominantly via 5-HT_{2C} receptor subtypes) thereby increasing circulating glucocorticoid levels (Heisler et al., 2007). In adrenalectomized rats, the fluoxetine-induced decrease of *bdnf* was of a similar magnitude compared to sham-operated rats, making the involvement of glucocorticoids in this effect unlikely (Table 2). Acute stress decreases hippocampal *bdnf* independently of adrenal steroids (Murakami et al., 2005). Hence, the influence of stress on fluoxetine-induced inhibition of *bdnf* in the present study cannot be fully excluded (Smith et al., 1995; Vaidya et al., 1999).

Pre-treatment with the selective 5-HT_4 antagonist, SB-204070 (Kennett et al., 1997) blocked the fluoxetine-induced reduction of hippocampal *bdnf* (Fig 2a). Consistent with this effect, the 5-HT_4 agonist renzapride (Dumuis et al., 1989) reduced *bdnf* levels, an effect also blocked by SB-204070 (Fig 2b). In contrast, pre-treatment with the selective 5-HT antagonists, WAY-100,635 or granisetron selective for 5-HT_{1A} and 5-HT_3, respectively (Barnes and Sharp, 1999; Fletcher et al., 1996; Maksay, 1996) failed to attenuate the inhibitory action by fluoxetine on *bdnf*. The 5-HT_2 antagonist ketanserin, previously shown to attenuate stress-induced reductions of *bdnf* (Vaidya et al., 1997), blocked the inhibitory action by fluoxetine in CA3 but not in the dentate gyrus in our study. In line with this, the 5-HT agonist DOI has been shown to dose-dependently decrease hippocampal *bdnf* (Vaidya et al., 1997).

Electrophysiological studies indicate the presence of excitatory 5-HT receptors on GABA-ergic interneurons innervating the granule cells in the dentate gyrus (Freund and Buzsaki, 1996; Piguet and Galvan, 1994). Taking into account the location of such 5-HT receptors, including 5-HT_4 types, combined with our recent finding that SSRI-induced reduction of hippocampal *bdnf* is blocked by pre-treatment with selective $GABA_B$ antagonists, suggests that the acute fluoxetine-induced inhibitory action on *bdnf* levels in the dentate gyrus is triggered by a functional interaction between 5-HT and GABA (Khundakar and Zetterstrom, 2011). In support of this, previous electrophysiological studies demonstrate that 5-HT, under some experimental conditions, acts via 5-HT_4 receptors to excite GABA-ergic interneurons in the hilus of the dentate gyrus (Bijak and Misgeld, 1997). Thus, the activation of excitatory 5-HT receptors (5-HT_4 and/or 5-HT_2) receptors, via fluoxetine-induced increases of extracellular 5-HT, could facilitate spontaneous GABA release *in vivo*, resulting in neuronal inhibition of the granule cells leading to reduced *bdnf* expression (Marmigere et al., 2003; Zafra et al., 1991).

Previous studies show that acute and chronic fluoxetine administration rapidly increases the phophorylation of the hippocampal BDNF receptor TrkB, suggesting an increase in BDNF release, which could lead an auto-inhibitory action on *bdnf* transcription (Sairanen et al., 2005). It is, however, unlikely that this effect is related to the fluoxetine-induced inhibition of *bdnf* in the present study; thus the transient action on TrkB phosphorylation has recently been reported to be independent of 5-HT transporter blockade, as well as BDNF signaling (Rantamaki et al., 2011).

As shown previously, twice-daily injections of fluoxetine for 14 days, at 24 hours after the last injection, significantly increased levels of *bdnf* in the dentate gyrus of the hippocampus, (Coppell et al., 2003; De Foubert et al., 2004; Khundakar and Zetterstrom, 2006; Nibuya et al., 1995). We thus next sought to determine the influence of 5-HT in producing this fluoxetine-induced up-regulation of *bdnf* levels in the dentate gyrus. Two weeks' fluoxetine treatment failed to increase *bdnf* levels in dentate gyrus of 5,7-DHT-lesioned rats compared to the corresponding lesioned saline controls. This suggests that fluoxetine relies on an intact 5-HT system to produce this effect. However, at four weeks after the lesion, levels of *bdnf* were significantly elevated (+ 20%) in lesioned rats compared to the corresponding sham controls and it remains unclear if chronic fluoxetine would be able to increase it further. An increased expression of hippocampal *bdnf* has also been detected following 5-HT depletion by *p*CPA (Zetterstrom et al., 1999). Enhanced BDNF produced in response to a toxic injury could promote the sprouting of surviving 5-HT neurons; indeed, 5-HT neurons express the gene for the tyrosine BDNF receptor Trkb (Madhav et al., 2001; Mamounas et al., 1995). Similarly, *bdnf* expression is enhanced following a lesion of the dopamine system with 6-hydroxydopamine (Zhou et al., 1996).

Preclinical data suggest a possible role for 5-HT_6 receptors in depression; however, both blockade and stimulation of this receptor evoke an antidepressant effect (Borsini et al., 2011). In support of previous biochemical and behavioral evidence for antidepressant effects of acute 5-HT_6 receptor stimulation (Nikiforuk et al., 2011; Svenningsson et al., 2007), we have previously shown that a single injection of the $5HT_6$ agonist LY-586713 increases hippocampal *bdnf*, an effect completely blocked by the selective $5HT_6$ antagonist, SB-271046 and of similar magnitude to those shown

after chronic, but not acute treatment, with fluoxetine (de Foubert et al., 2007). Here, we show that repeated injection of the 5-HT_6 agonist results in a time-dependent decline of its stimulatory action on hippocampal *bdnf*. Daily injections for 4 days of LY-586713, at 18 hour after the last injection, increased *bdnf* in CA1 and CA3 but not in the dentate gyrus (a non-significant trend only); however, after 14 days of treatment the drug failed to enhance hippocampal *bdnf* (Fig 3). The mechanism behind the time-dependent desensitized response by LY-586713 on hippocampal *bdnf* in the present study is not clear. It is, however, plausible that the acute stimulatory effect by the 5-HT_6 agonist on *bdnf* is mediated via increased cyclic AMP levels and the subsequent activation of CREB a known transcription factor for *bdnf,* and that repeated administration of LY-586713 leads to a desensitization of this effect. Indeed, a previous study demonstrates rapid agonist-induced desensitization of 5-HT_6 receptor-induced stimulation of adenylyl-cyclase in 5-HT_6 stably transfected HEK-293 cells (Max et al, 1995).

Commented [a4]: Which agonist? Write it in place of this text

The present study shows that daily administration of LY-586713 for 4 days significantly increased cell survival, but not cell proliferation, in dentate gyrus to a similar magnitude previously recorded after 2-3 weeks of antidepressant treatment (Malberg et al., 2000; Sairanen et al., 2005). In contrast, 14 days of LY-586713 treatment had no significant action on cell survival (Figs 4-6), reflecting its effect on *bdnf*. Taken together, these two separate findings relating to LY-586713 support the suggestion of desensitization of hippocampal 5HT_6 receptors following chronic agonist stimulation. Assuming that LY-586713-induced enhancement of *bdnf* expression leads to increased levels of the corresponding protein, it is interesting to note that a recent study shows that BDNF down regulates 5-HT_{2A} receptor protein

levels in hippocampal cultures. Interestingly, this effect was also shown to be time-dependent and not present until at least 7 days of BDNF exposure (Trajkovska et al., 2009). Thus, it can be speculated that increased BDNF levels following acute-sub chronic LY-586713 administration results in decreased 5-HT_6 protein levels.

In conclusion, the present study shows that the transient fluoxetine-induced down-regulation of *bdnf* expression depends on an intact 5-HT system and is primarily mediated via $5HT_4$ receptors. In comparison, the study found little support for a primary role of 5-HT_6 receptors in the mediation of chronic fluoxetine-induced up-regulation of *bdnf* expression and cell survival.

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Figure Legends

Fig 1. Effect of 5-HT lesioning by 5,7-DHT on fluoxetine (F, 10 mg/kg, i.p.)-induced inhibition of *bdnf* levels in dentate gyrus (white bars), CA1 (grey bars) and CA3 (black bars) of dorsal hippocampus at 4 hours after injection. Data are expressed as % of sham saline (S) controls and each column represents mean ± S.E.M. value from 5-6 rats. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to Sham (S) controls and $^{+}P < 0.05$, $^{++}P < 0.01$ compared to Sham (F), one-way ANOVA, with Bonferroni post-hoc analysis.

Fig 2. Effect of a 30 minutes' pre-treatment period with the 5-HT_4 receptor antagonist SB-204070 (SB, 1 mg/kg, i.p.) on a) fluoxetine- (Flu, 10 mg/kg, i.p.) and b) renzapride (Renz, 1 mg/kg, i.p.)-induced inhibition of *bdnf* levels in dentate gyrus (white bars), CA1 (grey bars) and CA3 (black bars) areas of dorsal hippocampus at 4 hours after the last drug administration. Data are expressed as % of saline (Sal)/saline treated controls and each column represents mean ± S.E.M. value from 5-6 rats. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to saline/saline controls and $^{+}P < 0.05$ and $^{++}P < 0.05$ compared to the corresponding saline/fluoxetine (a) or saline/renzapride (b) groups (one-way ANOVA, with Bonferroni post-hoc analysis).

Fig 3. Effect of 4 days' treatment (a) and two weeks (b) with the 5-HT_6 receptor agonists LY-586713 (1 mg/kg, s.c., daily injections) on *bdnf* levels in dentate gyrus (DG), CA1 and CA3 of dorsal hippocampus at 4 hours after the agonist administration. Data are expressed as % of saline treated controls and each column represents mean ± S.E.M. value from 5-6 rats. $^{**}P < 0.01$ compared to saline control (one-way ANOVA, with Bonferroni post-hoc analysis).

Fig 4. Representative photographic image of BrdU-labelling in the dentate gyrus (x 10 magnification) 28 days following daily administration of vehicle (a) and the 5-HT_6 agonist LY-586713, 1mg/kg, s.c. (b). Arrow indicates BrdU-labelled cells in the in the subgranular zone and granular cell layer SGZ/GCL.

Fig 5. Effect of daily injections of the 5-HT_6 receptor agonists LY-586713 (1 mg/kg, s.c.) for 4 days on total number of BrdU-positive cells 28 days after the last BrdU injection (measure of cell survival) in the subgranular zone and granular cell layer SGZ/GCL and the hilus of the denate gyrus. Each column represents mean ± S.E.M. value from 5-6 rats. **P* <0.05 compared to saline control (Student's un-paired t-test).

Fig 6. Effect of daily injections with the 5-HT_6 receptor agonists LY-586713 (1 mg/kg, s.c.) for 4 days or 14 days on total number of BrdU-positive cells 28 days after the last BrdU injection (measure of cell survival) in the dentate gyrus (total cell count for: SGZ/GCL and the hilus). Each column represents mean ± S.E.M. value from 5-6 rats. **P* <0.05 compared to the corresponding vehicle control groups (Student's un-paired t-test).

Table 1. Effect of noradrenaline lesioning by DSP-4 on fluoxetine (F, 10 mg/kg, i.p.) - induced inhibition of *bdnf* levels in dentate gyrus, CA1 and CA3 of dorsal hippocampus at 4 hours after the fluoxetine injection. Data are expressed as % of sham saline (S) controls and each column represents mean ± S.E.M. value from 5-6 rats. **P* <0.05, ***P* <0.01 compared to Sham (S) controls, one-way ANOVA, with Bonferroni post-hoc analysis.

Table 2. Effect of fluoxetine (Flu, 10 mg/kg, s.c.) at 4 hours after the injection on *bdnf* levels in: dentate gyrus, CA1 and CA3 of dorsal hippocampus in adrenoectomized (ADX) rats. Data are expressed as either percentage of sham ADX or saline (Sal) (ADX) and each column represent, mean ± S.E.M. value from 5-6 rats. **P* <0.05 and ***P* <0.05 compared to the corresponding Sal (ADX) control group, (one-way ANOVA, with Bonferroni post-hoc analysis).

Table 3. Effect of 30 minutes' pre-treatment with 5-HT receptor subtype selective antagonists: WAY-100,635 (0.3 mg/kg, s.c.), ketanserin (Ket, 5 mg/kg, i.p.), granisetron (Gran, 1 mg/kg, i.p.) on acute fluoxetine (Flu) induced reduction of hippocampal *bdnf* levels at 4 hours after the 2nd injection. Data are expressed as % of saline (Sal)/Sal treated controls and each column represents mean ± S.E.M. value from 5-6 rats. **P* <0.05 and ***P* <0.01, compared to saline/saline controls and $^{+}$*P* <0.05, compared to the corresponding Sal/Flu group (one-way ANOVA, with Bonferroni post-hoc analysis.