The Roles of BDNF, pCREB and Wnt3a in the Latent Period Preceding Activation of Progenitor Cell Mitosis in The Adult Dentate Gyrus by Fluoxetine

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Abstract

The formation of new neurons continues into adult life in the dentate gyrus of the rat hippocampus, as in many other species. Neurogenesis itself turns out to be highly labile, and is regulated by a number of factors. One of these is the serotonergic system: treatment with drugs (such as the SSRI fluoxetine) markedly stimulates mitosis in the progenitor cells of the dentate gyrus. But this process has one remarkable feature: it takes at least 14 days of continuous treatment to be effective. This is despite the fact that the pharmacological action of fluoxetine occurs within an hour or so of first administration. This paper explores the role of BDNF in this process, using the effect of a Trk antagonist (K252a) on the labelling of progenitor cells with the mitosis marker Ki67 and the associated expression of pCREB and Wnt3a. These experiments show that (i) Fluoxetine increased Ki67 counts, as well as pCREB and Wnt3a expression in the dentate gyrus. The action of fluoxetine on the progenitor cells and on pCREB (but not Wnt3a) depends upon Trk receptor activation, since it was prevented by icv infusion of K252a. (ii) These receptors are required for both the first 7 days of fluoxetine action, during which no apparent change in progenitor mitosis occurs, as well as the second 7 days. Increased pCREB was always associated with progenitor cell mitosis, but Wnt3a expression may be necessary but not sufficient for increased progenitor cell proliferation. These results shed new light on the action of fluoxetine on neurogenesis in the adult dentate gyrus, and have both clinical and experimental interest.
Transgenic mice with reduced BDNF levels or impaired TrkB signalling in the brain have provided evidence that TrkB activation is required for the behavioural actions typically induced by antidepressants [20]. Wnt signalling has been implicated in both embryonic and adult neurogenesis [21,22], but whether it is involved in the action of fluoxetine on adult neurogenesis is not known.

We have addressed the question of the importance of TrkB receptor activation on antidepressant effect on cell mitosis and on the latent period preceding activation of progenitor cell mitosis. The first experiment determines whether intracerebroventricular (icv) infusion of the Trk antagonist K252a, can block the action of fluoxetine (10 mg/kg/day) on progenitor cell mitosis, and the effect it has on the expression of pCREB and Wnt3a in the granular neurons in the dentate gyrus. The second explores whether rats treated with the same dose of fluoxetine for 7 days show any change in progenitor proliferation, or expression of pCREB or Wnt3a, compared to those treated for 14 days. Then the role of the Trk receptors in the latent period required for fluoxetine to act was investigated by giving K252a for either the first or second 7 days of the 14 day treatment period.

Materials and Methods

Animals

All procedures were carried out under Home Office (UK) licence. Male Sprague-Dawley rats 8 weeks old (Harlan, Oxon, UK) were used, weighing 200–250 grams at the start of the experiment. Rats were housed individually in a controlled environment. Ambient temperature was maintained at 21°C and humidity at 55% with ad libitum access to food and tap water. Animals were kept in a reversed 12-h light:12-h dark cycle, (lights off at 10.00 h).

Cannula placement

Animals were anaesthetised with isofluorane, oxygen and NO and placed securely into a stereotaxic frame (David Kopf instruments, Tujunga, CA, USA). A cannula (length 5 mm, outside diameter 0.36 mm; Charles River, Margate, UK) was implanted into the right lateral ventricle. Coordinates were 1 mm posterior and 1.5 mm lateral from bregma, –3.3 mm from the cortex [23]. The cannula was fixed in place by dental cement attached to the skull. They were connected to an Alzet osmotic minipumps (model 1002; volume 3.74 ml/cm (ID 0.69 mm). Since flow is 6 ml/day, the osmotic minipumps were attached to the tube 11.3 cm long and a volume of 3.74 ml/cm (ID 0.69 mm). Since flow is 6 ml/day, the tube will empty by day 7. This was checked in vitro at 37°C. In group (ii) both tubing and pump were filled with K252a; in group (iii) the tubing contained K252a, and the pump saline only; this was reversed in group (iv). All animals were killed on day 15 and the brains were snap frozen and placed in −70°C until they were sectioned (as above). Sections were stained for Ki-67.

Brain sections

Brains were sectioned in the coronal plane at 20 µm. The brain from each rat was sampled from −2.80 to −4.52 behind bregma. Sections were cut at −20°C using a Bright cryostat, and every sixth section was mounted on a polylysine-coated microscopic slide, six to a slide. The slides were left in a fume hood to dry overnight, then stored at −70°C. For each rat twelve sections through the dorsal hippocampus were analyzed 120 µm apart (ie one in six sections) for Ki-67 staining.

Immunohistochemistry

K67. Sections were incubated in 0.01 M citric acid for 40 min at 98°C. They were cooled and washed twice ×5 min in KPBS. Endogenous peroxidase activity was quenched with 3% H2O2 solution for 10 min followed two 5 min washes with KPBS. They were incubated with primary antibody (1:100 mouse monoclonal IgG anti-human Ki-67; Novocastra, Newcastle Upon Tyne, UK) and 1% horse serum in a humidified chamber at room temperature overnight. The next day, after twice ×5 min washes with KPBS, they were incubated with secondary antibody (1:200 biotinylated mouse IgG; Vector laboratories Ltd, Peterborough UK) for 1 hour at room temperature. After twice ×5 min washes with KPBS, they were incubated with Avidin-Biotin-Peroxidase reagent (Vector laboratories Ltd, Peterborough UK) for a further hour, followed by twice ×5 min washes with KPBS. The staining was visualized using DAB tablets (3,3-diaminobenzidin) (Sigma, Dorset, UK) for 5 min. Slides were then counterstained with 10% crystal violet solution followed by dehydration through ethanol and HistoClear. They were coverslipped with DPX for light microscopy and at ×40 magnification.

pCREB and Wnt3a. Three sections from each animal (1 in 12; between bregma −3.14 mm and −3.30 mm) were incubated in rabbit anti pCREB (Cell Signalling, Hitchen, UK; 1:25 in 0.5%...
Triton with 1% goat serum) overnight in a humidified chamber at room temperature. After two washes with KPBS, the sections were incubated in Alexa Fluor 568 (Invitrogen, Paisley, UK) goat anti-rabbit (1:200 in 0.5% Triton) for one hour, washed twice with KPBS, and cover slipped.

For single staining with Wnt3a, sections were incubated in rabbit anti-Wnt3a (Abcam, Science Park, Cambridge UK; 5 μg/ml in 0.5% Triton) overnight in a humidified chamber at room temperature. The following day, after two washes with KPBS, they were incubated in Alexa Fluor 568 goat anti-rabbit (1:200 in 0.5% Triton) for one hour, and washed twice with KPBS and covered slipped.

BDNF mRNA in situ hybridisation

Sections were allowed to air dry at room temperature and were then fixed with 4% paraformaldehyde (Sigma, Dorset, UK) for 5 min, washed in PBS and then dehydrated in 70% ethanol and 95% ethanol for 5 min before final storage in fresh 95% ethanol. In situ hybridization was carried out under RNAase-free conditions. The synthetic antisense oligonucleotide probe was confirmed by BLAST searches. A 48 base and a 45 base oligonucleotide complementary to exonic mRNA encoding BDNF mRNA [25]. 5' agt tcc agr gct tgt tga gcc cct gca gct tcc ttc gtg taa ccc g 3' was used. All probes were end-labelled with 32P-ATP as follows: 2 μl of purified oligonucleotide (5 ng/μl) was added to 1.25 μl Buffer and 1.25 μl calf thymus DNA (New England, BioSystem, UK). DEPC (Diethyl pyrocarbonate) was added to terminate the reaction. Purification of labelled probe from unincorporated nucleotides was accomplished by centrifugation through a G-50 Sephadex micro-column (Amersham, UK). Probes were evaluated for incorporation of radiolabel by scintillation counting. All hybridizations were carried out at 25°C–50°C cpm/μl in hybridization buffer (50% deionized formamide, 4× SSC, 5× Denhardt’s,100 μg/ml polyadenylic (potassium salt) acid, 200 μg/ml salmon sperm DNA, 120 μg/ml heparin (BDH, Leicestershire, UK), 25 mM sodium phosphate pH 7.0, 1 mM sodium pyrophosphate, 10% (w/v) dextran sulphate in DEPC-treated water (all Sigma, Dorset, UK). Sections were covered with parafilm and hybridized overnight at 44°C in a humid atmosphere. Excess unbound probe was removed using the following washes: 1× SSC (saline sodium citrate, Sigma, UK) at room temperature, 2×30 min at 55°C with 1× SSC and then rinsed at room temperature for 2 min, each in 1× SSC, 0.1× SSC, 18 Ω water, 50%, 70%, and 95% ethanol (BDH, Leicestershire, UK).

Sections were exposed to autoradiographic X-ray film (Amersham, Buckinghamshire, UK) for 14 days. Sense probes were run as negative controls.

Quantification

Proliferating cells (Ki67).

All slides were randomised and coded prior to quantitative analysis. Labelled cells were counted using a 40X objective; only cells on the internal border of the subgranular zone of the dentate gyrus were included. The data shown are the mean number of Ki67-positive cells in the SGZ of all sections per animal.

BDNF mRNA expression.

Sections and C14 labeled standards of known radioactivity (Amersham, Buckinghamshire, UK) were placed in X-ray cassettes and exposed to autoradiographic film. The optical density (OD) of the autoradiographic images was measured using a computerized PC-based image analysis system (NIH Image). ODs from the dentate gyrus from three consecutive sections per rat were obtained and averaged. The mean value for each rat was entered into the equation derived from the C14 standards and the final value was used to calculate group means. Sections from all groups were processed at the same time to avoid intrinsic variations between different in situ hybridizations.

Statistical analysis

Between-group one/two-way analysis of variance (ANOVA) and Bonferroni’s post hoc test were used when applicable. Log transformation was used to ensure homogeneity of variance before ANOVA when appropriate. Results were considered statistically significant if p<0.05.

Results

Experiment 1: Effect of K252a on the response of progenitor cells to fluoxetine

In this experiment, we tested whether blockade of the Trk receptor by icv K252a would prevent the stimulating action of fluoxetine on mitosis in the neurogenic area of the dentate gyrus.

Ki67. Ki67 positive cells were found in the SGZ of all animals including controls, confirming the presence of a basal level of progenitor cell mitosis in this region. There was a significant interaction between fluoxetine and K252a treatments (Two-way ANOVA, F (1,20) = 36.7, p<0.001). Icv K252a alone had no effect on the number of proliferating cells (Bonferroni, p>0.05). 10 mg/kg/day fluoxetine increased the number of proliferating cells by 80% (p<0.001) (Figure 1A). This effect was completely blocked by K252a (p>0.05). The number of Ki67-labelled cells in this group was not significantly different from the controls group (p>0.05), but was significantly less than after fluoxetine treatment alone (p<0.005) (Figure 1A).

BDNF mRNA. K252a alone had no effect (F = 0.28, p/ns). Fluoxetine increased levels, as expected (F = 34.6, p<0.001), but this was not prevented by simultaneous infusion of K252a; there was thus no significant interaction between K252a and fluoxetine (F = 2.4, pns) (Figure 1B).

pCREB. Saline-treated animals showed little expression of pCREB protein in the dentate gyrus. This was markedly stimulated by fluoxetine treatment. K252a-treated animals showed a similar picture to the saline-treated group (Figure 1C). Adding K252a to fluoxetine resulted in reduced pCREB expression which now resembled animals treated with saline.

Wnt3a. Saline-treated animals showed little expression of Wnt3a protein in the dentate gyrus (Figure 1D). This was markedly stimulated by fluoxetine treatment. K252a-treated animals showed a similar picture to the saline-treated group. However, adding K252a to fluoxetine did not prevent the latter’s stimulating effect on Wnt3a expression (Figure 1D).

In summary: this experiment showed that icv K252a prevented the stimulating effect of fluoxetine on progenitor cell mitosis in the dentate gyrus, but did not prevent increased BDNF mRNA. There was a selective action of K252a on inhibiting the effect of fluoxetine on pCREB but not Wnt3a.

Experiment 2: Effect of treatment with K252a for either the first or second 7 days of a 14 day fluoxetine treatment

Since fluoxetine treatment is required for at least 14 days to increase progenitor cell mitosis rates, we asked whether activation of Trk receptors was required for the whole of this period.
The number of Ki-67 cells increased significantly as expected after 14 days of fluoxetine treatment (Figure 2A). However, at 7 days there was no difference between fluoxetine and control groups (two-way ANOVA: fluoxetine x time F = 14.0, p = 0.002).

BDNF. BDNF mRNA expression showed a similar pattern. Expression increased after 14 days in the fluoxetine-treated group, but not 7 days, (F = 150, p < 0.001) (Figure 2B). Pairwise comparisons showed that BDNF levels at 7 days were no different between the fluoxetine-treated and control groups (Bonferroni, p > 0.05) but that fluoxetine treatments at 7 and 14 days were significantly different from each other (p < 0.001) (Figure 2E, F).

pCREB and Wnt3a. There was also no observable change in either pCREB or Wnt3a at 7 days, but by 14 days the expression of both was clearly increased (Figure 2C, D).

Ki-67. The number of Ki-67 cells increased significantly as expected after 14 days of fluoxetine treatment (Figure 2A). However, at 7 days there was no difference between fluoxetine and control groups (two-way ANOVA: fluoxetine x time F = 14.0, p = 0.002).

In summary. The first part of this experiment showed that 7 days treatment with fluoxetine had no effect on Ki67 labelled cells, or on the expression of either pCREB or Wnt3a. However, all three were accentuated after 14 days treatment. The second part showed that blocking Trk receptors with icv K252a for either the first or second 7 day period of a 14 day fluoxetine infusion prevented the expected increase in progenitor cell mitosis. As in experiment 1, pCREB was reduced in fluoxetine and K252a-treated animals, but increased expression of Wnt3a still occurred in fluoxetine-treated animals given K252a.

Table 1 summarises the results of these two experiments on the expression of Ki67, pCREB and Wnt3a in the dentate gyrus.

Discussion

These experiments further define the role of BDNF in the regulation of the mitosis rates of progenitor cells in the dentate gyrus by serotonin-acting drugs such as fluoxetine. Progenitor mitosis rates are the essential first step in the overall control of neurogenesis in the adult hippocampus, since they determine its early stages. Other factors then regulate the differentiation, survival and connectivity of newly-formed neurons [26]. Labelling cells with Ki67 is now well established as a reliable indicator of progenitor cell mitosis in the dentate gyrus, and is highly correlated with BrdU uptake [27]. The first experiment reported here established that blockade of Trk receptors by the drug K252a was able to prevent entirely the stimulating action of fluoxetine on mitosis of the progenitor cells. The K252 family of alkaloid toxins are protein kinase inhibitors, and K252a has a long history of being used to antagonise Trk receptors, with particular affinity for TrkB [28,29,30]. Icv K252a has already been shown to prevent...
some actions of BDNF on serotonin release after SSRI administration [31], but there has been no report on its ability to inhibit SSRI-induced progenitor cell mitosis. Although we cannot be entirely certain that our results reflect specific blockade of TrkB, the accumulative evidence points strongly in this direction.

K252a did not prevent the fluoxetine-induced increase in BDNF mRNA expression, suggesting that this is upstream of the action of TrkB receptors, as might be expected. However, the results on pCREB were clear-cut: fluoxetine increased its expression and this was prevented by K252a. Since increased pCREB may be an essential step in the up-regulation of progenitor mitosis [32], it is interesting that this occurred not just in the cells adjacent to the neurogenic niche in the inner layer, but throughout the dentate gyrus. This suggests that the function of CREB in the dentate gyrus may be more complex than was originally thought, and that cells other than those in the neighbourhood niche may also contribute to the control of neurogenesis [7]. In contrast, the expression of Wnt3a, also known to be closely involved in neurogenesis [3,22,33,34] was, as expected, increased by fluoxetine, but this was not prevented by K252a. This experiment therefore shows that functional activity of Trk, and hence – it may be presumed – BDNF is essential for the stimulating action of fluoxetine on progenitor cell mitosis. This agrees with results on BDNF-knockout mice, in which SSRIs and similar drugs have reduced actions [35,36]. However, these studies were limited to heterozygous knock-outs only, since homozygous ones do not survive. It is interesting that K252a by itself did not decrease progenitor mitosis below baseline: this may reflect the fact that its blockade of TrkB may have been incomplete, or that BDNF is required only for above-basal proliferation rates. K252a also blocked the fluoxetine-induced expression of pCREB, supporting the role for this compound in the control of neurogenesis. However, it did not prevent increased Wnt3a expression, suggesting that the latter is not a sufficient event for increased progenitor cell mitosis.

The second experiment began by confirming, under our conditions, that a 7-day treatment with fluoxetine had no discernible effect on progenitor mitosis, or pCREB and Wnt3a expression, whereas after 14 days all three were stimulated. We use osmotic minipumps to deliver fluoxetine, since we (and some others) find that parenteral injections are not effective [37,38]. The striking result, however, was that blocking TrkB for either the first or the second 7 days of a 14 day treatment with fluoxetine prevented increased progenitor mitosis. It is during the second period that BDNF mRNA expression is increased, so it might seem logical that blockade only during this time would prevent the BDNF-dependent action of fluoxetine. Had this occurred it would have implied that the latent period preceding the onset of fluoxetine’s action was due to some event upstream of the action of BDNF. But this was not the case. It seems clear that BDNF is required throughout the latent period, though future experiments

![Figure 2. The effect of either 7 or 14 days treatment with fluoxetine (10 mg/kg/day). A: Ki67 cell counts, B: BDNF mRNA expression, C: pCREB D: Wnt3a. E,F: in situ hybridization images of BDNF mRNA. Values as in Figure 1. Bar represents 100 μm. Values are mean ± SEM.**p<0.001 compared to control. Representative sections are shown from fluoxetine-treated animals only, since there is virtually no expression in controls. doi:10.1371/journal.pone.0013652.g002](image-url)
might explore this more precisely, using more frequent sampling points. Despite the fact that BDNF mRNA was increased only after 14 days of fluoxetine treatment, blocking its action at a time when there was no discernible change in BDNF mRNA prevented not only the expected increase in progenitor mitosis, but also that in pCREB, presumably a more proximal regulator of neurogenesis. The conclusion must be that BDNF may be active throughout the latent period. However, we have shown previously that icv infusions of BDNF are effective after 7 days [39], so the latent period is not due to a delayed response to BDNF. Our experiments do not yet allow us to offer a precise explanation of these results, but there may be two processes. The first, dependent on ‘basal’ BDNF, may represent one that, in some way, allows increased BDNF to occur at some time after 7 days from initiation. The second part of this process may depend on heightened BDNF production, and it is this that results in increased pCREB expression, and thus enhanced progenitor mitosis. Some part of this whole process is highly sensitive both to the absolute levels of corticoids as well as their circadian pattern [26,39,40,41,42].

Our results suggest that the expression of pCREB is downstream of the action of BDNF, since it was prevented by K252a. Since CREB has a range of intracellular actions, these may not all link with Wnt3a. Wnt3a has been implicated in the ability of human neural progenitors to form neurospheres in culture [33] and in the process of adult neurogenesis, though its exact role is still unclear [43,44]. Our results suggest that factors additional to Wnt3a (maybe other members of the Wnt family) may be needed to increase progenitor cell mitosis rates. Table 1 shows that, whilst we find a consistent association between pCREB and increased mitosis, this was not true for Wnt3a. However, increased Wnt3a may be an essential ingredient of the stimulatory process, since we never observed one without the other, and inhibition of Wnt signalling reduces adult neurogenesis in the dentate gyrus [3]. Our results further suggest that the stimulating action of fluoxetine on Wnt3a may not be dependent on BDNF (or

Figure 3. Effect of treatment with K252a icv for either the first or second 7 days of a 14 day fluoxetine (10 mg/kg/day) treatment.
A: Ki67-labelled cell counts; B: Wnt3a expression in the dentate gyrus. Values as in Figure 1. Bar represents 100 μm.
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Trk receptors; we have not investigated the lower-affinity p75 receptor), so there may be other pathways linking the action of this drug to neurogenesis in the dentate gyrus. The ability of fluoxetine to increase Wnt3a is consistent with this drug’s reported induction of neural plasticity [45,46,47]. This may well contribute to its efficacy as an anti-depressant.

The experiments reported here emphasise the central role of BDNF in the regulation of mitosis of the progenitor cells in the dentate gyrus of the adult rat, and its contribution in the latent period preceding the effect of fluoxetine on this process. The role of BDNF in the expression of pCREB and Wnt3a may be distinct. Our results further suggest that the control of the neurogenic niche may be widespread throughout the dentate gyrus, though the identity of the cells that produce BDNF is still obscure. They do not add directly to the evidence linking adult neurogenesis with depression, but strengthen understanding of the role of BDNF and its receptor in the action of anti-depressants such as fluoxetine.

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Author Contributions

Conceived and designed the experiments: SBP JH. Performed the experiments: SBP AMB NJP JH. Analyzed the data: SBP AMB NJP JH. Wrote the paper: SBP JH.

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