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Brain-derived neurotropic factor and neurogenesis in the adult rat dentate gyrus: interactions with corticosterone

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Abstract

Flattening the diurnal corticosterone rhythm prevented the stimulating action of L-NAME (a nitric oxide synthase, NOS, inhibitor) on progenitor cell proliferation in the dentate gyrus in Lister-Hooded adult male rats. The increased expression of brain-derived neurotrophic factor (BDNF) and trkB mRNA in the dentate gyrus which otherwise occurred after L-NAME was also prevented by clamping the corticoid rhythm in adrenalectomized rats, but was restored by daily additional injections of corticosterone (which replicates the diurnal rhythm). Unilateral infusions of BDNF into the lateral ventricle increased proliferation in the dentate gyrus on the side of the infusion, but this was not observed following implantation of subcutaneous corticosterone, which flattened the diurnal corticosterone rhythm. 5HT1A mRNA in the dentate gyrus was increased on both sides of the brain by unilateral BDNF infusions, but this was also prevented by subcutaneous corticosterone pellets. These results show that the diurnal rhythm of corticosterone regulates the stimulating action of NOS inhibitors on BDNF as well as on neurogenesis in the dentate gyrus, and that BDNF becomes ineffective on both proliferation rates and 5HT1A expression in the absence of a rhythm in corticosterone. This, together with our previous findings, suggests that corticoid rhythms permit both serotonin and NO access to BDNF, and the latter to regulate progenitor cell activity.

Keywords

brain-derived neurotrophic factor; corticosterone; dentate gyrus; neurogenesis; nitric oxide; serotonin

Introduction

Neurogenesis in the dentate gyrus of the adult hippocampus is highly labile. Much of this lability is due to the exquisite sensitivity of the progenitor cells to glucocorticoids (Gould *et al.*, 1992; Wong & Herbert, 2004). Glucocorticoids have two principal roles: absolute levels regulate the proliferation rate of the progenitor cells; and the presence of an intact diurnal corticoid rhythm is an essential requirement for the action of two further controlling factors, namely serotonin and nitric oxide (NO). The stimulating actions of both the selective serotonin-reuptake inhibitor (SSRI) fluoxetine and the nitric oxide synthase (NOS) inhibitor L-NAME are prevented if the diurnal rhythm of corticosterone is flattened by either implanting a pellet of corticosterone into intact rats, or maintaining adrenalectomized rats on a constant supply of corticosterone (Huang & Herbert, 2006; Pinnock *et al.*, 2007). Restoring the diurnal rhythm in the latter by an additional daily injection of corticosterone

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also reinstates sensitivity of their progenitor cells to either drug (Huang & Herbert, 2006; Pinnock *et al.*, 2007).

Brain-derived neurotrophic factor (BDNF) has an established role in the regulation of proliferation in the dentate gyrus. BDNF has high levels of expression in the adult dentate gyrus (Nibuya *et al.*, 1995; Berchtold *et al.*, 1999). The action of BDNF is mediated by the specific tyrosine kinase receptor TrkB, which is also widely expressed in the dentate gyrus (Barbacid, 1994). L-NAME as well as markedly stimulating neurogenesis has also been found to increase BDNF in the dentate gyrus (Cheng *et al.*, 2003). BDNF is also sensitive to other treatments that increase neurogenesis, such as SSRIs (Nibuya *et al.*, 1995; Duman *et al.*, 1997; Nakagawa *et al.*, 2002). Chronic but not acute SSRI treatment upregulates BDNF mRNA and its receptors in the hippocampus (Nibuya *et al.*, 1995). Furthermore, infusions of BDNF into the hippocampus and midbrain area produce antidepressant-like behavioural effects in rats (Shirayama *et al.*, 2002) and transgenic mice with reduced BDNF signalling in the brain are insensitive to antidepressants in behavioural tests (Sairanen *et al.*, 2005). Activation of 5HT1A receptors by 8-hydroxy-2-(di-n-propylamino)tertraline (8-OH-DPAT) increased progenitor cell mitosis (Huang & Herbert, 2005), but whether these receptors are required for the action of SSRIs on progenitor cell proliferation is uncertain (Santarelli *et al.*, 2003; Holick *et al.*, 2008).

These experiments raise several questions: does BDNF respond to L-NAME and, if so, is this dependent on an intact diurnal rhythm of corticosterone? If this were so, it might indicate that NO acted via BDNF to increase neurogenesis, and that this interaction was corticosterone-sensitive. The second question is whether the action of BDNF itself is dependent on the corticosterone rhythm. Would direct infusions of BDNF into the brain increase neurogenesis in the absence of such a rhythm? Furthermore, given the role of 5HT1A, would the same provisos apply to this class of serotonin receptor? BDNF infusions into the brain upregulate proliferation (Scharfman *et al.*, 2005). Fluoxetine increases BDNF mRNA expression in the dentate gyrus, but only after around 14 days of treatment, a period also required for significant increments in the number of dividing progenitor cells (Altieri *et al.*, 2004; De Foubert *et al.*, 2004; Sairanen *et al.*, 2005). NO can increase both neuronal differentiation and BDNF levels in cultures of embryonic cortex (Cheng *et al.*, 2003).

In the experiments reported here, we explore the way that BDNF interacts with corticosterone in the regulation of the rate of proliferation of progenitor cells in the dentate gyrus. First, we show that injections of L-NAME upregulate the expression of BDNF and trkB mRNA. We go on to show that L-NAME is ineffective on BDNF in adrenalectomized (ADX) rats maintained on a constant dose of cortisosterone, but that the sensitivity of BDNF mRNA expression to L-NAME was restored by giving these rats a daily injection of corticosterone, which also restores their diurnal rhythm. We also show that intra-cerebroventricular (i.c.v.) infusions of BDNF stimulated progenitor proliferation on the side of the infusion only. They also markedly increased the expression of 5HT1A receptors (on both sides). Neither increased proliferative activity nor 5HT1A expression was observed in rats implanted with a corticosterone pellet (which flatten the diurnal rhythm), thus suggesting that sensitivity of the proliferative process to this growth factor and its regulation of 5HT1A receptors is moderated by glucocorticoids.

Materials and methods

Animals

All procedures were carried out under Home Office (UK) licence. Male Lister-Hooded rats (Harlan, Oxford, UK) were used; they weighed around 200-250 g at the start of the experiment. Rats were housed in groups of three per cage in a controlled environment except

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for experiment 3 in which the animals were caged individually. Ambient temperature was maintained at 21 °C and humidity at 55% with *ad libitum* access to food and tap water (and 0.9% saline for ADX animals). Animals were kept on reversed 12/12-h light-dark cycles (lights off at 10.00 h).

Experimental manipulations

Implants of corticosterone—In-house corticosterone pellets were prepared by melting cholesterol and corticosterone together at a ratio of 70 : 30. Each pellet weighed 200 mg.

Cannula placement—Animals were anaesthetized 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, -3.5 mm depth from cortex (Paxinos & Watson, 1998). The cannula were fixed in place by dental cement attached to two stainless steel screws inserted into the skull and connected to an Alzet osmotic minipump (model 1007D; volume 100 μL, flow rate 0.5 μL/h; Charles River) via medical grade vinyl tubing (6 cm length). All pumps were implanted subcutaneously in the posterior upper thorax and were filled the day before surgery. The pumps and tubing were filled with either recombinant human BDNF (1 μg/μL; Invitrogen, Paisley, UK) dissolved in phosphate-buffered saline (PBS) with 0.5% rat serum albumin (RSA) or PBS. They were incubated at 37 °C overnight in a sterile saline solution to prime them before implantation. Animals received 12 μg/day of recombinant human BDNF for 7 days (Pencea *et al.*, 2001). Animals were weighed daily. The position of each cannula was assessed post-mortem by examining its track on sections stained with cresyl violet. Animals were anaesthetized with isoflurane, oxygen and NO injecting a terminal dose of pentobarbitone sodium.

In all experiments, blood samples were collected by cardiac puncture at 10.00 h (i.e. at the start of the dark phase) into heparinized syringes within 3 min of injecting a terminal dose of pentobarbitone sodium, centrifuged and stored at -20 °C until assayed for plasma corticosterone. Brains were collected, frozen immediately on dry ice and then stored at -70 °C until required.

Experimental groups

Experiment 1

The effect of giving L-NAME (daily injection) on the expression of BDNF and trkB mRNA in intact rats: This experiment explored the effect of the NOS inhibitor L-NAME on the expression of BDNF mRNA and trkB in the dentate gyrus. Two groups of 12 rats received daily intraperitoneal injections of either 0.9 saline (control) for 7 days or L-NAME (50 mg/kg dissolved in 0.9% saline). All animals were killed 24 h after the last injection and blood samples taken for corticosterone (10.00 h). The expression of BDNF and trkB mRNA was examined using *in situ* hybridisation.

Experiment 2

Effects of l-NAME on the expression of BDNF mRNA in ADX rats implanted with a 30% corticosterone pellet: This experiment tested whether BDNF mRNA expression following L-NAME was inhibited by ‘clamping’ plasma corticosterone, and whether this could be restored by adding daily corticosterone injections.

Twenty-four rats were adrenalectomised and implanted subcutaneously with a single 30% corticosterone/cholesterol pellet. The next day half of the animals received a daily injection

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of either corticosterone subcutaneously (2 mg/kg) or sesame oil at the beginning of the dark phase (10.00 h) for 12 days (n = 5 per group). Five days later half of each group received either a daily injection of 50 mg/kg L-NAME (dissolved in 0.9% saline) or a control injection (saline) for a further 7 days. All animals were killed 2 h after the last injection of L-NAME (10.00 h) and blood samples taken for corticosterone. Sections were stained for BDNF mRNA as above but also for glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA.

Experiment 3

Effect of BDNF infusion into the lateral ventricle in intact rats treated with a 30% corticosterone pellet: One group of ten intact rats were implanted with a subcutaneous cholesterol pellet and a second group of 14 with a single 30% corticosterone/cholesterol pellet. Half of each of these two groups were also implanted with osmotic minipumps filled either with recombinant human BDNF with added 0.5% RSA or PBS with 0.5% RSA (see above). These pumps were attached to a cannula inserted into the right lateral ventricle. Seven days later all animals were killed and blood samples were taken for corticosterone assay (10.00 h); sections were stained for (1) Ki-67 or (2) 5HT1A receptor mRNA.

Brain sections

Coronal sections were taken from the entire length of the dorsal hippocampus and mounted on poly-lysine microscopic slides (BDH, Leicestershire, UK) and stored in -70 °C until required. Several series of sections, each one in six of those cut, were taken. All measurements were made on 12 sections for Ki-67, and three sections for *in situ* hybridization (see below for further details).

Corticosterone assay

Plasma corticosterone concentrations were measured by radioimmunoassay according to a validated procedure described previously (Chen & Herbert, 1995). The intra-assay coefficients of variation were: 5.1% for experiment 1, 6.2% for experiment 2 and 4.5% for experiment 3. The sensitivity of the assay was 0.98 ng/mL.

Immunohistochemistry

Sections for all the immunostainings were first fixed for 5 min in 4% paraformaldehyde (pH 7.4, Fisher, Loughborough, UK) then rinsed twice with K-PBS. For Ki-67 immunohistochemistry, sections were incubated in 0.01 M sodium citrate buffer (pH 6.0) for 40 min at 98 °C, rinsed, treated with hydrogen peroxide for 10 min, rinsed and then incubated overnight in K-PBS containing 1% normal horse serum, 0.5% Triton and a mouse monoclonal antibody against Ki-67 (1 : 100; Novocastra, Newcastle upon Tyne, UK). The sections were incubated with biotinylated secondary mouse IgG antibody and visualized with avidin-biotin-peroxidase complex, followed by diaminobenzidine reaction. They were then dehydrated by passing through graded alcohols and incubated in Histoclear overnight, and cover slipped under DPX for light microscopic analysis.

In-situ hybridization

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 finally storing in fresh 95% ethanol. *In-situ* hybridization was carried out under RNAase-free conditions. The accuracy of synthetic antisense oligonucleotide probes was confirmed by BLAST searches on the NCBI database. A 48-base and a 45-base oligonucleotide complementary to exonic mRNA encoding BDNF and trkB mRNA (Al-Majed *et al.*, 2000) 5′-

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AGTTCCAGTGCCTTTTGTCATGCCCCTGCAGCTTCCTTCGTGTAACCC-3′ and 5′-GAGAGGGCTGGCAGAGTCATCGTCGTTGCTGATGACGGAAGCTGG-3′ were used. For 5HT1A mRNA the probe was 5′-GGTTAGCGTGGGAGGAAGGGAGACTAGCTGTCTGAGCGACATACAAG-3′ (Fairchild *et al.*, 2003). For GR the probe was 5′-AGGAGAATCCTCTGCTGCTTGGAATCTGCCTGA-3′ (McQuade *et al.*, 2004), and for MR was 5′-TTCGGAATAGCACCGGAAACGCAGCTGACGTTGACAATCT-3′ (van Riel *et al.*, 2003)

All probes were end-labelled with ^{35}S-ATP as follows: 2 μL of purified oligonucleotide (5 ng/μL) was added to 1.25 μL buffer and 1.25 μL cobalt chloride (New England Biosystem, Hitchin, Hertfordshire, UK). DEPC-treated water (6.5 μL) was added, followed by 1 μL terminal ^{35}S deoxyadenosine 5′ (α-thio) triphosphate (10 mCi/mL; Amersham, Bucks, UK) and 0.5 μL (15-20 U) terminal deoxynucleotide transferase enzyme (New England Biosystem). Probes were incubated at 37 °C for 1 h before 40 μL of DEPC was added to terminate the reaction. Purification of labelled probe from unincorporated nucleotides was accomplished by centrifugation (2092.5 ***g*** for 2 min) 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 2500-5000 c.p.m./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), 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: slides were rinsed in 1× SSC (Sigma) at room temperature, washed twice for 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). Sections were thoroughly air-dried at room temperature before exposure to autoradiographic X-ray film (Amersham) for 7 days for BDNF, 14 days for trkB and 6 days for 5HT1A mRNA. Sense probes for each of the mRNAs were run as negative controls.

Quantification

Proliferating cells—All slides were randomized and coded prior to quantitative analysis. Sections were examined using a 40× objective. Ki-67-labelled stained cells were counted under experimentally 'blind' conditions. Only cells on the internal border of the subgranular zone were included. The data shown are the mean count per section obtained from 12 sections per animal.

mRNA expression—For quantification of mRNA, the sections and ^{14}C-labelled standards of known radioactivity (Amersham) were placed in X-ray cassettes and then exposed to autoradiographic film. The optical density (OD) of the autoradiographic images was measured using a computerized PC-based image analysis system (NIH Image, North Carolina, USA). ODs were obtained from three consecutive sections per rat and the mean value for each rat was entered into the equation derived from the ^{14}C standards. The final value was used to calculate group means. Sections from all groups were hybridized at the same time to avoid intrinsic variations between different *in situ* hybridizations.

Statistics

Results were analysed using one- or two-way ANOVA where appropriate. Data were transformed if the variances of the means were not homogeneous. Pairwise comparisons were by made using the Bonferroni test unless otherwise indicated.

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Results

The effect of l-NAME on expression of BDNF and trkB mRNAs in the dentate gyrus of intact rats

The number of Ki-67-labelled cells was markedly increased by L-NAME [$F = 18.6$, $P < 0.001$; reported previously (Pinnock *et al.*, 2007) - data not shown]. L-NAME also significantly increased the expression of both BDNF and trkB mRNAs in the dentate gyrus ($F = 467.5$, $P < 0.001$; $F = 179.4$, $P < 0.001$; Fig. 1).

Effects of l-NAME on expression of BDNF mRNA in ADX rats implanted with 30% corticosterone pellets and given an additional daily injection of corticosterone (2 mg/kg)

The mean plasma level of corticosterone in these animals has already been reported (Pinnock *et al.*, 2007). It should be noted that there was no significant difference between L-NAME-treated ADX rats given an extra daily dose of corticosterone and those receiving saline (Bonferroni: $P > 0.05$).

There was a highly significant interaction between L-NAME and corticosterone treatments ($F = 35.1$, $P < 0.001$) and further analysis, using pairwise comparisons, showed that whereas L-NAME had no effect on BDNF in ADX rats receiving a daily saline injection (Bonferonni: n.s.), there was a highly significant increase ($P < 0.001$) in those given daily additional corticosterone (2 mg/kg) which reconstituted the diurnal rhythm (Fig. 2).

There was a significant interaction between additional daily injections of corticosterone and L-NAME on GR ($F = 9.8$, $P = 0.008$) but not on MR mRNA ($F = 0.5$, $P =$ n.s.). Pairwise comparisons showed that MR was decreased by L-NAME (Bonferroni, $P = 0.02$) but not by daily additional corticosterone, whereas GR was unaffected by L-NAME but increased by daily corticosterone ($P < 0.001$; Table 1).

Effect of BDNF infusions into the right lateral ventricle in intact rats with or without flattened diurnal corticosterone rhythms

One animal implanted with corticosterone and infused with BDNF died. Plasma corticosterone levels of the remainder are shown in Table 2. All values fell within the range of those expected at the start of the dark phase, and there were no significant differences between the four groups.

Overall, BDNF increased Ki-67 cell counts on the right (infused) side ($F = 85.7$, $P = 0.022$) but not on the other side ($F = 0.05$, $P =$ n.s.; two-way ANOVA). Corticosterone reduced Ki-67 cell counts in both the left and the right (BDNF-infused) dentate gyrus compared with control (right side: $F = 8.13$, $P = 0.009$; left: $F = 4.99$, $P = 0.035$; Fig. 3). There were no significant interactions between BDNF and corticosterone treatments. However, a pair-wise analysis showed that whereas BDNF significantly increased Ki-67 counts on the infused side in control animals (without a corticosterone pellet; $F = 6.01$, $P = 0.04$) this was not the case in the corticosterone-implanted animals ($F = 1.37$, $P =$ n.s.). There were no significant changes on the other (non-infused) side in either group (one-way ANOVA).

Overall, 5HT1A receptor mRNA reflected a highly significant interaction between BDNF and corticosterone on both the right (BDNF-infused) side ($F = 19.85$, $P < 0.001$) and the left ($F = 16.75$, $P = 0.001$) (two-way ANOVA). BDNF increased 5HT1A mRNA on both sides (right: $F = 16.5$, $P = 0.001$; left: $F = 7.2$, $P = 0.016$), but corticosterone had no first-order effect (right, $F = 2.1$; left, $F = 1.3$; both $P =$ n.s.). (Fig. 4). A pair-wise analysis confirmed that BDNF increased 5HT1A mRNA on both sides in animals receiving a cholesterol pellet (right: $F = 35.2$, $P < 0.001$; left: $F = 20.7$, $P = 0.002$). Repeated-measures analysis showed a

greater response to BDNF on the infused side than on the other ($F = 7.02$, $P = 0.029$). However, BDNF had no effect on either side in animals treated with corticosterone (right, $F = 0.08$; left, $F = 1.1$; both P = n.s.).

Discussion

Three significant findings were evident from the present study. L-NAME, a drug that is well established as a generalised inhibitor of NOS, has a powerful stimulating action on progenitor cell mitosis rates in the adult dentate gyrus (Packer *et al.*, 2003; Park, 2003; Pinnock *et al.*, 2007), which is prevented by administering an NO donor (Cheng *et al.*, 2003). It also increases BDNF and trkB (its principal receptor) mRNA expression. A previous report, focused principally on the effects of exercise and with no measure of progenitor cell proliferation, and using a different dose of L-NAME, found a nonsignificant increase in BDNF alone (Chen *et al.*, 2006). We show that this action, like that on the progenitor cells (Pinnock *et al.*, 2007), is dependent on the presence of a diurnal rhythm in corticosterone, as it is not observed in ADX rats given a replacement corticosterone regime that replicates levels similar to that in intact rats, but lacking diurnal variation. Reinstating this rhythm by giving an additional, small, daily injection of corticosterone also reinstates the ability of L-NAME to increase BDNF mRNA. We measured the expression of both MR and GR to determine whether changes in these receptors might correlate with the effects of L-NAME or the efficacy of additional daily corticosterone. There was a main effect of L-NAME on MR; as this receptor type has been implicated in progenitor cell proliferation (Wong & Herbert, 2005), this might contribute to the actions of L-NAME on neurogenesis. However, the effects on GR were more complex, and the two treatments interacted. Although it might seem surprising that additional corticosterone increased GR expression in the dentate gyrus, we have found a similar result after 7 days of treatment of intact rats with higher daily doses of corticosterone (40 mg/kg; our unpublished data). It is evident that the two types of receptor react differentially to corticosterone (Conway-Campbell *et al.*, 2007).

Given that it seems clear that both L-NAME and fluoxetine, a drug acting on serotonin, require the presence of an intact corticosterone rhythm to be effective, and both increase BDNF expression (Sairanen *et al.*, 2005; present study), a major question was whether the action of BDNF itself also depended on the same rhythm. Our results show clearly that it does, as i.c.v. infusions of BDNF increased the number of Ki-67-labelled cells - an established index of mitotic activity, and one that correlates closely with BrdU labelling (Kee *et al.*, 2002; Pinnock *et al.*, 2007) - in the dentate gyrus after 7 days treatment in intact rats, but not in those whose daily rhythm had been flattened by a subcutaneous implant of corticosterone. Increased 5HT1A mRNA expression was also prevented by flattening the corticoid rhythm, a procedure that diminishes 5HT release in response to SSRIs (Gartside *et al.*, 2003a).

It is very important to distinguish two actions of glucocorticoids on neurogenesis in the adult dentate gyrus, although it is not always so easy to separate them. The original observation that neurogenesis is markedly suppressed by increased levels of glucocorticoids (Cameron & Gould, 1994) has been amply confirmed, and extended to show that survival of newly generated neurons is also impaired (Wong & Herbert, 2004). Flattening the diurnal rhythm does not, in general, alter progenitor cell activity, although it should be noted that maintaining corticosterone levels near, say, normal peak values may actually increase overall exposure of the brain to glucocorticoids, as the usual daily nadir will be eliminated. However, an intact diurnal rhythm seems essential for alterations either in serotonin (e.g. induced by treatment with an SSRI such as fluoxetine) or in NO (following L-NAME treatment) to activate progenitor cells in the adult dentate gyrus (Huang & Herbert, 2006; Pinnock *et al.*, 2007). These major regulatory factors thus depend on an intact diurnal

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glucocorticoid rhythm for their action. In contrast, the activity of the neurogenetic niche in the dentate gyrus is highly sensitive to absolute levels of glucocorticoids.

There is considerable evidence that BDNF may be implicated in the control of neurogenesis in the adult hippocampus. This area is the site of active axonal growth and synaptogenesis, as well as the formation of new neurons, and BDNF is known to play a central role in these processes (Monteggia *et al.*, 2004) as well as in plastic alterations in the function of the hippocampus (e.g. long-term potentiation; Lu, 2003; Bramham & Messaoudi, 2005; Thomas & Davies, 2005). More specifically, treatments that increase neurogenesis (e.g. anti-depressant drugs such as fluoxetine, exercise) also increase BDNF (Dias *et al.*, 2003; Adlard *et al.*, 2004; Altieri *et al.*, 2004; De Foubert *et al.*, 2004; Sairanen *et al.*, 2005). BDNF infusions stimulate progenitor cell mitosis (Scharfman *et al.*, 2005; present study). Exposure to stress or to excess glucocorticoids, which depress neurogenesis, have been generally found to decrease BDNF (Schaaf *et al.*, 1998; Hellsten *et al.*, 2002; Rasmusson *et al.*, 2002; Dwivedi *et al.*, 2006; Hansson *et al.*, 2006; Prickaerts *et al.*, 2006), although there have been exceptions (Chao *et al.*, 1998). These findings give rise to two further questions: is the regulation of BDNF by other regulatory factors dependent on the diurnal corticoid rhythm? And is its action on neurogenesis also moderated by the diurnal glucocorticoid rhythm? These are the questions addressed herein.

We show that whereas L-NAME stimulates both progenitor cells and BDNF in control rats, these are prevented by flattening the glucocorticoid rhythm, further reinforcing the putative link between stimulation of proliferation and increased BDNF expression. The essential role of the diurnal corticoid rhythm was confirmed in ADX corticosterone-implanted rats (which, of course, have no corticoid rhythm), and in which a daily additional injection of corticosterone (which restores this rhythm) also reinstated the effect L-NAME had on BDNF mRNA expression. It also, as we show elsewhere, enables L-NAME to activate the progenitor cells (Pinnock *et al.*, 2007). If, however, the role of the diurnal corticoid rhythm was simply to enable alterations in NO or serotonin to increase BDNF (and hence stimulate the progenitor cells), then i.c.v. infusions of BDNF itself would bypass this control gate and still be effective even when the corticoid rhythm had been flattened. But this is not what we found. BDNF was unable to increase Ki-67 counts in rats in which corticosterone had been implanted subcutaneously, a procedure previously shown to flatten the daily rhythm (Gartside *et al.*, 2003a,b; Huang & Herbert, 2006). In our experiments, the mean levels of corticosterone were not altered by this procedure, which argues against the actions of BDNF being inhibited by excess corticoid, although we did find that flattened rhythms in this experiment reduced overall levels of Ki-67-labelled cells. The caveat mentioned above regarding overall exposure of the brain to glucocorticoid under these conditions should be borne in mind. We conclude that the action of BDNF on progenitor cell mitosis is also gated by the diurnal rhythm in corticoids.

In view of the role of serotonin in the regulation of neurogenesis, our finding that BDNF increased the expression of 5HT1A mRNA is interesting. Agonists of this receptor increase progenitor cell proliferation (Huang & Herbert, 2005), although whether the response to SSRIs such as fluoxetine is preserved in mice with deleted 5HT1A receptors seems uncertain (Santarelli *et al.*, 2003; Holick *et al.*, 2008). Whereas there is considerable literature on BDNF in the hippocampus being increased by drugs acting on serotonin (see above), the converse is not true (Deltheil *et al.*, 2007), although BDNF-deficient mice may have decreased 5HT1A activity (Daws *et al.*, 2007) and interactions between serotonin and BDNF have been found to predict depression (Kaufman *et al.*, 2006). In the present study, unilateral BDNF infusions increased 5HT1A mRNA expression in the dentate gyrus on both sides of the brain. But there was no associated increase in Ki-67 labelling on the contralateral side, suggesting that increased receptor expression by itself (without increased

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agonist activity) is not sufficient to stimulate progenitor cell division. However, just as a flattened corticosterone rhythm prevented the activating action of BDNF on progenitor cell division, so it did on the ability of BDNF to stimulate levels of 5HT1A mRNA. Is seems likely therefore that actions of BDNF on the brain in addition to neurogenesis may be dependent on the prevailing rhythm or levels of corticoids. What we do not yet know is whether these receptors are required for BDNF to increase neurogenesis.

Our results, together with those of earlier studies, allow us to generate a partial model of the control of the rate of proliferation of progenitor cells in the dentate gyrus. The regulating actions of at least two major control systems (serotonin and NO) are themselves subject to regulation by the presence of an intact diurnal corticoid rhythm, and this may limit access to activation of BDNF. The latter may represent at least part of the final common path of the control exerted by these two factors, but is itself subject to the corticoid rhythm. The mechanism responsible for this gating action is not yet known, but might involve rhythm-sensitive target genes such as the *per* clock genes, which are known to be expressed in the hippocampus, and are sensitive to corticoids (Hastings *et al.*, 2003).

The results here do not add directly to the evidence linking neurogenesis in the dentate gyrus with the clinical phenomenon of depression, although they do suggest additional approaches for further research. Altered neurogenesis has been suggested to contribute to depression (Jacobs *et al.*, 2000), and to its recovery following treatment with anti-depressants (Santarelli *et al.*, 2003). BDNF is also thought to play a role in the occurrence of, or recovery from, depression (Kalueff *et al.*, 2006; Hilt *et al.*, 2007; Martinowich *et al.*, 2007). Whether response to anti-depressants depends both on BDNF and, in turn, on current patterns (rhythms) of daily cortisol remains to be investigated. It is striking that higher levels of cortisol predispose to depression (Goodyer *et al.*, 2000; Harris *et al.*, 2000), that diurnal rhythms are often disturbed during the illness (Bridges & Jones, 1966), and that genetic polymorphisms in both serotonin and BDNF have been implicated in the risk for this condition (Caspi *et al.*, 2003; Kaufman *et al.*, 2006; Castren *et al.*, 2007).

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Abbreviations

ADX	adrenalectomized
BDNF	brain-derived neurotropic factor
i.c.v.	intra-cerebroventricular
NO	nitric oxide
NOS	nitric oxide synthase
OD	optical density
RSA	rat serum albumin
SSRI	selective serotonin-reuptake inhibitor

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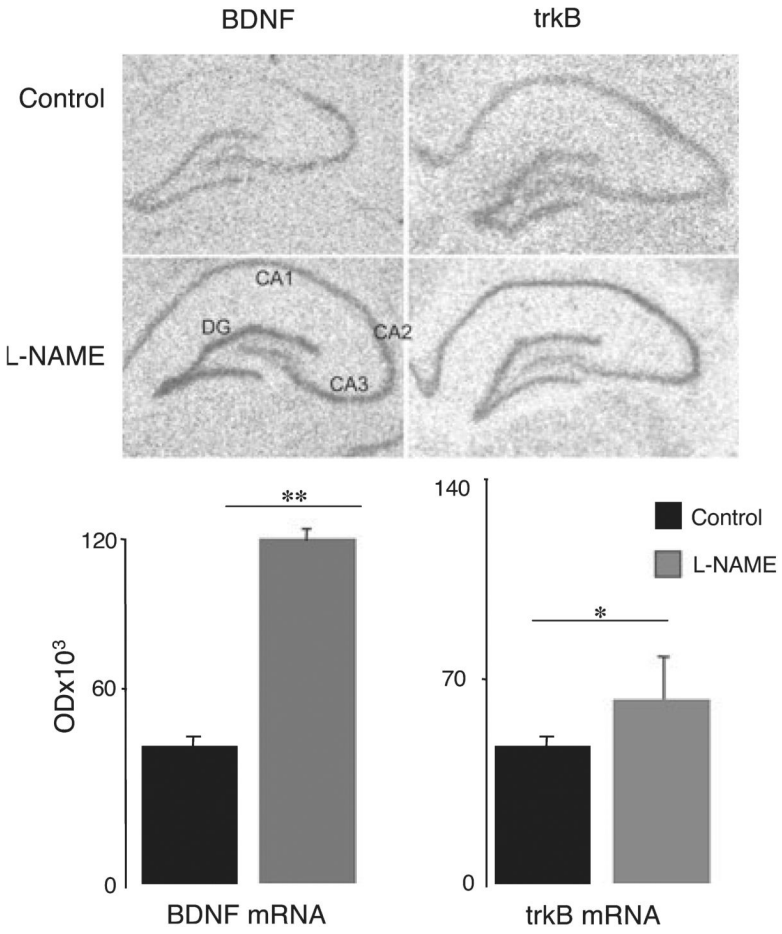

Fig. 1.

Expression of BDNF and trkB mRNA in the dentate gyrus of intact rats following treatment with L-NAME (50 mg/kg/day) for 7 days. Above: sections showing expression of the two mRNAs by *in situ* hybridization. Below: effects on optical density values in the dentate gyrus. Values are mean ± SEM. $*P < 0.05$, $**P < 0.001$ compared with control.

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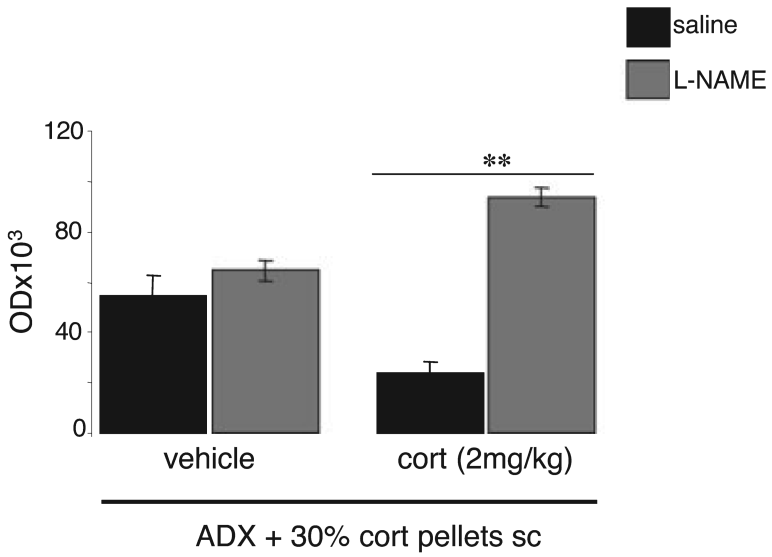

Fig. 2.

Effect of L-NAME on BDNF mRNA expression in adrenalectomised (ADX) Lister-Hooded rats implanted with one 30% corticosterone pellet and receiving either additional (2 mg/kg/day) corticosterone or seasame oil daily at 10.00 h for 12 days. Values are mean ± SEM. ** $P < 0.001$ compared with controls.

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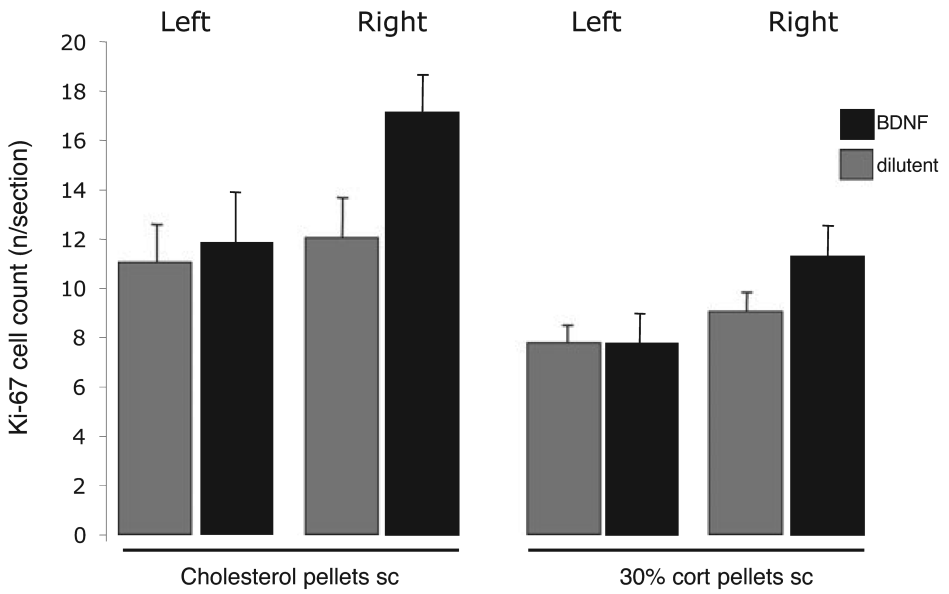

Fig. 3.

Effect of right-sided intra-cerebroventricular (i.c.v.) infusions of BDNF for 7 days on Ki-67 cell counts (mean ± SEM) in the left and right dentate gyrus in rats implanted subcutaneously with either cholesterol, or one 30% corticosterone pellet. Significance levels given in the text.

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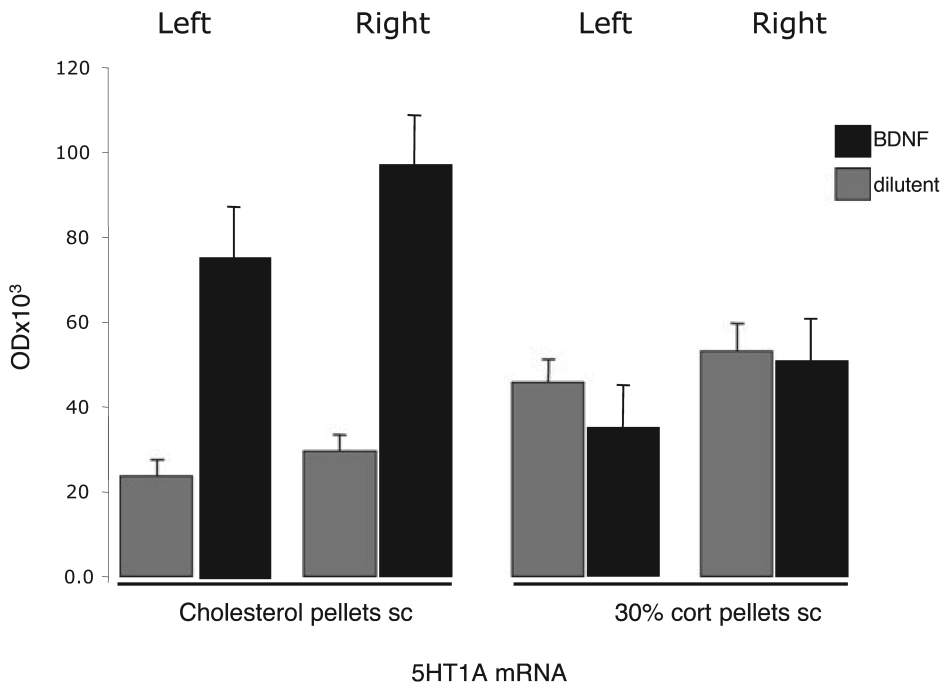

Fig. 4.

Effect of right-sided intra-cerebroventricular infusions of BDNF for 7 days on the level of 5HT1A receptor mRNA in the dentate gyrus in intact rats implanted with either cholesterol pellets or one 30% corticosterone pellets. Values are mean optical density ± SEM. Significance levels are given in the text.

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Table 1

GR and MR mRNA in the dentate gyrus in ADX corticosterone-implanted rats treated with either L-NAME or daily injections of additional cortocosterone or both

Treatment (in addition to ADX + 30% corticosterone pellet s.c.)	Optical density (pixels)	
	GR mRNA	MR mRNA
Oil s.c. + saline i.p.	112.6 ± 3.05	304.9 ± 32.57
Oil s.c. + L-NAME (50 mg/kg)	113.4 ± 2.60	244.2 ± 28.60
Daily cort (2 mg/kg) + saline i.p.	126.0 ± 2.36	277.7 ± 21.93
Daily cort (2 mg/kg) + L-NAME (50 mg/kg)	119.2±1.65	233.6±10.15

Data are presented as mean ± SD; Significance levels: Pairwise comparisons (Bonferroni): $*P < 0.05$, $**P < 0.01$ compared to control group.

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Table 2

Plasma levels of corticosterone in rats implanted either with a cholesterol or a 30% corticosterone pellet s.c., and infused i.c.v. with either BDNF or solvent

Treatment	*n*	Plasma corticosterone (ng/mL)
Saline (+RSA) i.c.v. + cholesterol s.c.	5	187.2 ± 36.5
Saline (+RSA) i.c.v. + corticosterone s.c.	9	141.0 ± 45.4
BDNF i.c.v. + cholesterol s.c.	5	132.4 ± 72.6
BDNF i.c.v. + corticosterone s.c.	8	130.0 ± 23.6

Data are presented as mean ± SD; *n*, number of animals in each group.