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European Journal of Pharmacology 583 (2008) 115–127
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Chronic low dose corticosterone exposure decreased hippocampal cell proliferation, volume and induced anxiety and depression like behaviours in mice

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Received 16 October 2007; received in revised form 17 December 2007; accepted 14 January 2008
Available online 26 January 2008

Abstract

A dysregulated hypothalamic-pituitary-adrenal axis (HPA) has been implicated in major depressive disorder and most commonly used animal models of depression have been shown to elevate circulating levels of plasma corticosterone. We have compared the effects of chronic and acute corticosterone administration on hippocampal cell proliferation (as measured by BrdU immunohistochemistry), hippocampal volume and the appearance of anxiety (light dark box) and depression (forced swim test) like behaviours in CD1 mice. We have also examined the effects of chronic administration of fluoxetine and imipramine on these parameters. Chronic (14 days) but not acute treatment with corticosterone resulted in reduced hippocampal cell proliferation and granule cell layer volume, these changes were prevented by co-administration of imipramine and fluoxetine. In contrast, acute and 7 day but not 14 or 21 day treatment with corticosterone gave rise to a "depressed" phenotype in the forced swim test. Mice treated for 14 days with corticosterone also developed an anxious phenotype in the light dark box but only upon repeated testing. The results presented here demonstrate that moderately elevated corticosterone for a prolonged period is sufficient to induce cellular changes in the hippocampus that are prevented by chronic administration of antidepressants.

Keywords: Glucocorticoid; Corticosterone; Neurogenesis; Depression; Antidepressant; Behaviour

1. Introduction

There is a close link between the incidence of major depressive disorder and dysregulation of the hypothalamic pituitary adrenal axis (HPA axis) (Rubin et al., 1987; Holsboer and Barden, 1996) including elevated circulating cortisol, the circadian regulation of cortisol secretion (Peeters et al., 2004) and impaired glucocorticoid receptor negative feedback of the HPA axis (glucocorticoid resistance) in depressed patients (Lowy et al., 1984). Such observations in conjunction with the fact that prolonged stress (characterised by activation of the HPA axis) is a major risk factor for the development of major depressive disorder (Shores et al., 1992) have lead to a great deal of research into the mechanisms by which HPA axis dysregulation may influence depressive disorders including the use of exogenous corticosteroid administration to re-capitulate aspects of this clinical situation and to investigate how antidepressants may affect the HPA axis in this abnormal state (Cameron et al., 1998; Fone and Topham, 2002).

Furthermore there are a number of similarities between features of depression and chronic glucocorticoid administration in laboratory animals. It has been reported that chronic exposure to corticosterone results in ventricular enlargement and a reduced hippocampal volume (Sapolsky, 2001; Sapolsky, 1985) a phenomenon which has also been reported in patients suffering from major depressive disorder (Sheline et al., 1996). Interestingly, in patients with Cushing's syndrome (a relatively rare hormonal disorder characterised by hypercortisolaemia) a reduction in hippocampal volume is also frequently seen (Starkman et al., 1992)

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doi:10.1016/j.ejphar.2008.01.014

and is normalised following the reduction in circulating cortisol levels (Starkman et al., 1999). There are clear links between the administration of glucocorticoids and reduced cognitive ability (Lupien et al., 1994), a trend seen in some depressed patients (Belanoff et al., 2001).

Most of the commonly used animal models of depression such as restraint stress, psychosocial stress, forced swimming and exposure to predator odour elevate circulating levels of corticosterone (Czeh et al., 2002; Harvey et al., 2003; Rittenhouse et al., 2002). Correspondingly, exogenous administration of high doses of corticosterone induce changes in behaviour, neurochemistry and brain anatomy (Baez and Volosin, 1994; Hill et al., 2003) which may be indicative of or consistent with a depressive like phenotype. Studies from several laboratories have also shown that chronic but not acute treatment with antidepressants increased hippocampal neurogenesis (Malberg et al., 2000) and that chronic psychosocial stress (Czeh et al., 2001) and chronic corticosterone treatment (Hellsten et al., 2002) caused deficits in hippocampal neurogenesis which were reversed by antidepressant or electroconvulsive therapy. It has also been shown that disrupting hippocampal but not subventicular zone neurogenesis blocked the behavioural effects of antidepressants in several rodent models (Santarelli et al., 2003).

The use of exogenously administered corticosterone has some validity as a model to study chronic stress or conditioned fear in animals based on several published findings including comparable effects on hippocampal neurogenesis (Karishma and Herbert, 2002), brain monoamine metabolism (Inoue and Koyama, 1996) and enhancement of depression like behaviours in assays such as the forced swim test. The relative role of glucocorticoid and mineralocorticoid receptors have also been explored (Wong and Herbert, 2005) demonstrating that agonists at both glucocorticoid receptors and mineralocorticoid receptor can reduce levels of hippocampal progenitor proliferation. Studies have shown that flattening of the corticosterone rhythm by implanting slow release corticosterone pellets changes the functional responsiveness of 5-HT_{1A} receptors similar to those seen in depressed patients (Leitch et al., 2003).

In this study we have compared the effects of chronic and acute corticosterone administration firstly on hippocampal cell proliferation (as quantified by bromodeoxyuridine (BrdU) immunohistochemistry), hippocampal volume and secondly on the appearance of anxiety and depression like behaviour. The effects of chronic administration of fluoxetine and imipramine on the cellular changes induced by corticosterone were also examined.

2. Materials and methods

2.1. Animals

Male CD1 mice (Charles River U.K.) weighing 22–24 g at the start of the experiment were used for all studies. All animals were group housed in solid bottomed perspex cages in groups of 4–5 in a temperature and humidity controlled room (lights on 07:00–19:00) with food and water available *ad libitum*. All animal studies were carried out between 8 a.m. and 11 a.m. to avoid any confounding results that may have been obtained due to conducting experiments during the hours when rodent plasma corticosterone rises sharply (usually between 12 p.m. and 8 p.m.). In all studies 6–8 mice were used per treatment group. Animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.

2.2. Drug formulation

For chronic studies pellets (Innovative Research of America, 21 day release pellet) consisting of corticosterone (5 mg/pellet) in a proprietary matrix of cholesterol, lactose, celluloses, phosphates, and stearates to facilitate a continuous and sustained release were used. Corresponding placebo pellets consisted of the same matrix. For acute corticosterone dosing studies it was decided to use the same dose equivalent as the highest daily release formulation i.e. 40 mg/kg. Comparable doses of corticosterone have also been used in rodent neurochemical and cognition studies (Inoue and Koyama, 1996; Pavlides et al., 1993).

For acute corticosterone dosing studies corticosterone (Sigma) was dissolved in absolute ethanol at a concentration of 40 mg/ml by sonicating using a sonic probe. The solution was then diluted to 4 mg/ml with the addition of sesame oil (Sigma). The resulting solution was sonicated for approximately 1 h in a sonicating water bath to ensure all corticosterone was in solution, as described elsewhere (Karishma and Herbert, 2002). Vehicle treated animals received the same 10% ethanol in sesame oil preparation as was used to prepare the corticosterone, all animals were dosed intraperitoneally (i.p.). Mice received the treatments in a dose volume of 10 ml/kg. Fluoxetine (Medicinal Chemistry Department, Terlings Park U.K.) and imipramine (Sigma) were formulated in saline at a concentration of 1 mg/ml (free base equivalent) and dosed i.p. in a dose volume of 10 ml/kg. BrdU (Sigma) was formulated in saline at a concentration of 5 mg/ml and dosed i.p. in a dose volume of 10 ml/kg.

2.3. Determination of plasma corticosterone concentration and adrenal gland weights

Terminal blood samples were collected into heparinised tubes (Beckton Dickinson), centrifuged at 1000 ×g. for 10 min, plasma collected and frozen at −80 °C until ready for analysis. Samples were analysed using the Coat-a-Count corticosterone RIA kit (DPC products). Adrenal glands were also removed, frozen on dry ice and stored at −80 °C until ready for analysis.

2.4. Determination of hippocampal cell proliferation

2.4.1. Corticosterone administration

Male CD1 mice weighing 23–25 g at the start of the experiment were briefly anaesthetised using isofluorane. Animals were implanted with 1, 2 or 4 × 5 mg corticosterone pellets or the corresponding number of placebo pellets giving the equivalent of 10, 20 or 40 mg/kg/day of corticosterone respectively. Following pellet implantation mice were returned to their home cage to recover for either 7, 14, 21 or 28 days (the final timepoint representing a 7 day washout following corticosterone). On the

last 5 days of each implant period mice were given a single daily 50 mg/kg i.p. injection of BrdU. 24 h later animals were terminally anaesthetised and brains were analysed for BrdU positive cells and hippocampal volume measurements as outlined below. In a separate study mice were injected with either corticosterone (40 mg/kg, i.p.) or vehicle (10% ethanol in sesame oil, i.p.) and returned to their home cage for 24 h. After this period all animals were given a single injection of BrdU (50 mg/kg, i.p.) and returned to their home cage. Two hours following BrdU injection animals were terminally anaesthetised, perfused and tissue processed for quantification of BrdU positive cells as outlined below.

2.4.2. Identification and quantification of BrdU positive cells

2 or 24 h after the last injection of BrdU animals were terminally anaesthetised using Euthatal (10 ml/kg i.p.) and were perfused transcardially with PBS followed by cold 4% paraformaldehyde in PBS (pH 7.4) (these two time points show different baseline cell numbers due to the different survival times for newborn cells with the 2 h period being subject to fewer labelled cells but more likely to exhibit greater degree of survival than the 5× daily dosing paradigm). Brains were removed and placed into 4% paraformaldeheyde in PBS (pH 7.4) for post fixing (7 days at 4 °C). Following postfixing in paraformaldehyde, brains were transferred to 30% sucrose in paraformaldehyde and kept at 4 °C until brains no longer floated in the solution, this prevents ice crystals forming when sectioning thus retaining the cellular morphology. Forty micron thick coronal brain sections were cut on a freezing microtome and every section was collected through the hippocampus, placed in a separate well of a 96 well plate containing Walter's antifreeze and samples were stored at −20 °C until ready for analysis. To ensure that the same neurone was not counted in two consecutive sections every fourth section was transferred to staining baskets and for BrdU staining.

Following four PBS rinses to remove all traces of antifreeze, sections were first incubated in 50% formamide/saline sodium citrate at 65 °C for 2 h (to denature DNA) followed by four PBS rinses and then transferred to 2 N HCl at room temperature for 30 min. The sections were then incubated in a borax wash (0.05 M boric acid, 0.0125 M sodium borate, pH8.5 with 0.6 ml Tween 20 per 200 ml) solution for 10 min followed by four PBS rinses. Sections were then placed in 3% hydrogen peroxide for 30 min to remove any endogenous peroxidases followed by four PBS rinses. Sections were then placed in blocking solution (5% normal rabbit serum (NRS)) for 1 h. The sections were incubated with rat anti-BrdU primary antibody (Oxford Biotechnology 1:200 dilution in 5% NRS) overnight at 4 °C with shaking. Sections were then washed four times in PBS and incubated with a biotinylated secondary anti-rat IgG secondary antibody in 5% NRS (Vector laboratories 1:200) for 1 h. Following incubation in ABC solution for 1 h, immunoreactive nuclei were visualized by incubation in DAB solution for 5 min followed by a rinse in de-ionized water. Sections were counterstained in 0.01% cresyl violet for 10 s followed by 2 rinses in de-ionized water to allow the dentate gyrus to be identified more easily.

2.4.3. BrdU quantification

BrdU positive nuclei were quantified using light microscopy (40× magnification) and stereological analysis. A modified unbiased stereology protocol was used that has been reported (Malberg et al., 2000) to successfully quantitate BrdU labelling. To ensure that all BrdU positive cells in the dentate gyrus were able to be quantified, sections were collected starting at the level of the fimbria hippocampus (Bregma—0.80 mm) and 40 μm sections collected through to the dorsal hippocampal commisure (Bregma—4.80 mm) which resulted in the collection of approximately 100 sections. Two major considerations in stereological analyses are that no BrdU-labelled cells be counted twice and that the area counted be consistent in each section. All BrdU-labelled cells in the dentate gyrus (subgranular zone of the granule cell layer) were counted in each section blinded to the study code. To distinguish single cells within clusters, all counts were performed at 40× magnification. A cell was counted as being in the subgranular zone of the dentate gyrus if it was touching or in the subgranular zone. Cells that were located more than two cells away from the subgranular zone were classified as hilar. The total number of BrdU-labelled cells per section was determined and multiplied by 4 to obtain the total number of cells per dentate gyrus.

2.4.4. Volumetric analysis

Sections used to stain for BrdU positive cells were also used to estimate granule cell layer and hippocampal volumes. This was achieved by using Cavalieri's Principle which states that if, in two solids of equal altitude, the sections made by planes parallel to and at the same distance from their respective bases are always equal, then the volumes of the two solids are equal (Kern and Bland, 1948). This is a principle which has been adapted for stereology as a method of volume estimation (McNulty et al., 2000). The method involved capturing digital images of all sections and drawing round either the whole hippocampus or the granule cell layer. An estimated 3D volume based on a set section thickness and set section interval was then calculated using the software package (AIS 6.0 Image Analysis Software, Imaging Research Inc.).

2.4.5. Antidepressant treatment

Mice were implanted with either corticosterone or placebo pellets as described above (40 mg/kg/day) and returned to their home cage to recover. 24 h after pellet implantation mice were dosed with either saline (10 ml/kg i.p.), imipramine (10 mg/kg i.p.) or fluoxetine (10 mg/kg i.p.), this was repeated daily for the duration of the experiment. On days 10–14 mice were given a single daily injection of BrdU (50 mg/kg i.p.). 24 h later animals were terminally anaesthetised and brains were analysed for BrdU positive cells and hippocampal volume measurements as outlined above.

2.5. Effects of corticosterone administration on mouse behaviour

The corticosterone administration schedule was as outlined above for cell proliferation studies.

2.5.1. Forced swim test

Mice were tested by placing in a glass cylinder (height = 25 cm, diameter = 10 cm) containing water (24–25 °C) to a depth of 14 cm. Swim, escape and immobility behaviours were monitored for a 5 min period using a Videotrack tracking system (Viewpoint, France) based on video image analysis. Parameters were defined to distinguish swimming, escape and immobility behaviours, a curve was generated for each animal in proportion to the general animal activity. At the end of an experiment, the activity count and duration of each type of movement was calculated.

2.5.2. Light dark box

The light dark box (also sometimes called the black white box) was described by Crawley (1981). Following the relevant dosing regimen mice were placed in the test box (45×27×27 cm) that was divided by a partition. The smaller chamber was coloured black and the larger chamber white. Between the two was a partition with a 7.5 cm×7.5 cm opening that allowed mice to move between compartments. The white compartment was illuminated, giving approximately 450 lx while the dark side was illuminated by red light to facilitate tracking with an infrared camera. Mice were placed individually into the white area, facing away from the black section and the time spent in the white section observed by remote video recording for 5 min. Time spent in each compartment as recorded using HVS Image VP200 tracker boxes and HVS Field software, software also recorded average speed in each compartment by dividing distance travelled by time spent moving. The HVS tracker system detects colour contrast i.e. black on white or vice versa, the system could easily detect the mice in the dark section of the box but was unable to detect the mice in the light side so a small black mark was placed on the animals back using a marker pen just before testing.

2.6. Statistical analysis

For all results normal probability plots of the data indicated that there was no deviation from standard statistical assumptions of normality. For BrdU quantification, volumetric analysis, plasma corticosterone levels and adrenal gland weights all data were analysed by two way ANOVA using treatment and either dose or duration of treatment as categorical predictors. In the case of studies consisting of only two groups Student's *t*-test analysis was used. For behavioural studies either two way ANOVA as described above or repeated measures ANOVA. Post hoc analysis was either by Dunnett's or Fisher's LSD test.

3. Results

3.1. The effects of corticosterone administration on plasma corticosterone concentration and adrenal gland weight

Determination of plasma corticosterone concentration by RIA showed that following an acute dose of corticosterone (40 mg/kg, i.p.), plasma levels were approximately 850 and 1700 ng/ml, 1 and 2 h following administration respectively as compared to 100 ng/ml in vehicle treated animals (Fig. 1A). In animals

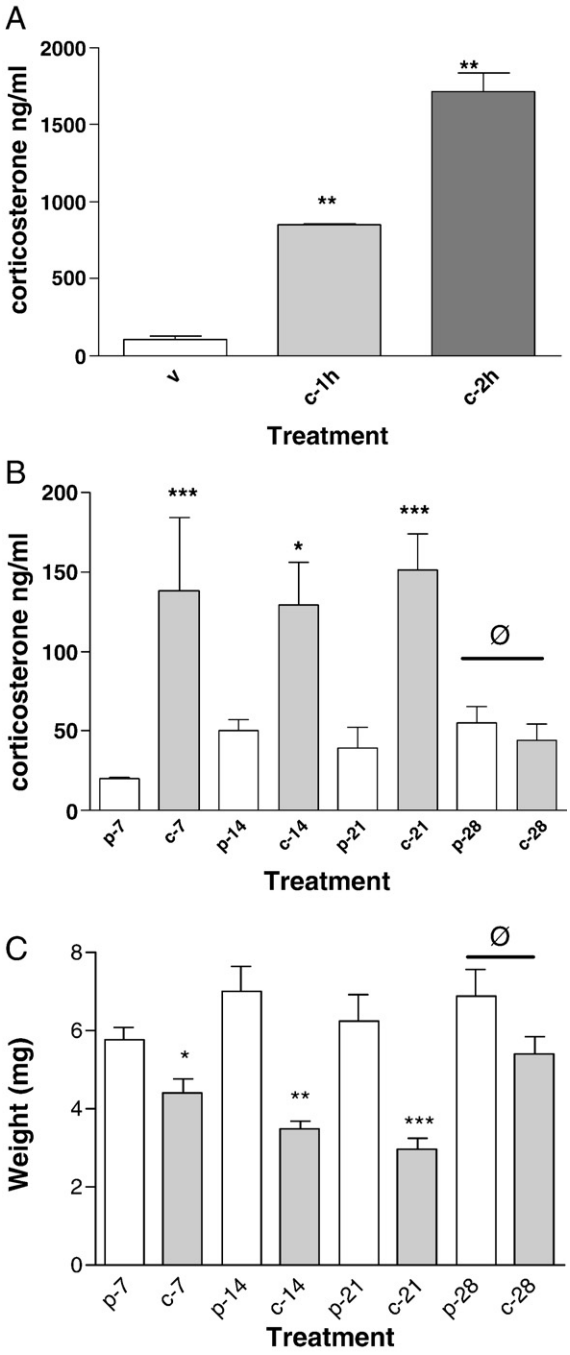

Fig. 1. The effects of chronic and acute corticosterone administration on plasma corticosterone concentration and adrenal gland weights. Plasma corticosterone levels were determined by RIA either (A) 1 or 2 h after i.p. administration of 40 mg/kg corticosterone or (B) following 7, 14, 21 or 28 days of pellet implantation. The 28 day time point represents 7 days with no significant corticosterone release from implanted pellets. (C) Adrenal glands were removed, frozen and weighed to determine the effects of chronic corticosterone administration on the natural synthesis and release of corticosterone by the adrenal glands. (v) vehicle (p) placebo (c) corticosterone (∅) no exogenous corticosterone released. Results shown are mean±s.e.m. with $n=6$–8 mice per treatment group.*$P<0.05$, **$P<0.01$, ***$P<0.001$ as determined by two way ANOVA with Fisher's LSD post hoc analysis (chronic studies) or one way ANOVA with Dunnett's post hoc analysis (acute studies). Note different *y*-axis scales.

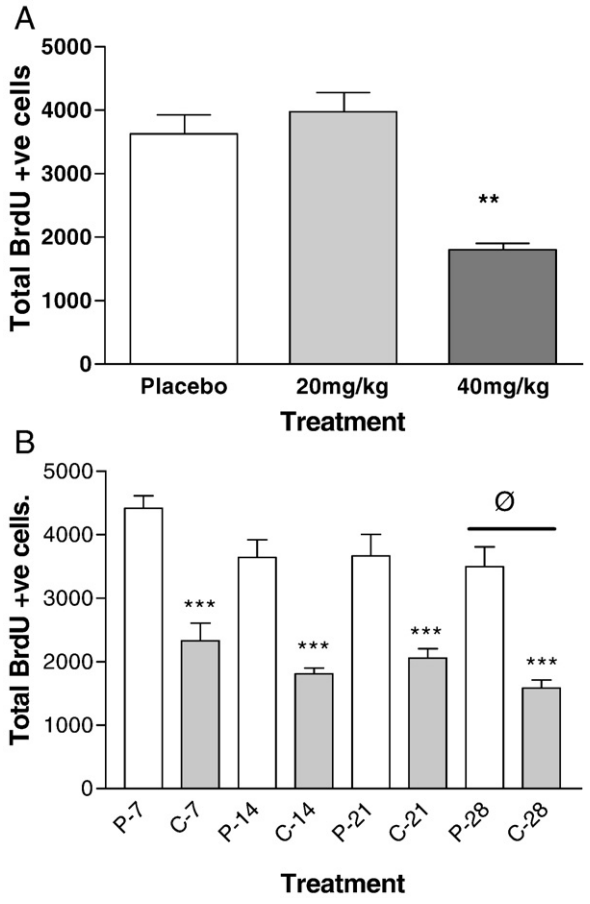

Fig. 2. Time Course of 40 mg/kg/day Corticosterone Treatment on Hippocampal Cell Proliferation. Hippocampal cell proliferation was quantified using BrdU immunohistochemistry following A) 14 day implant of placebo or corticosterone pellets releasing an equivalent of either 20 or 40 mg/kg/day of corticosterone or B) 7, 14, 21 or 28 day implant of placebo or corticosterone pellets releasing an equivalent of 40 mg/kg/day (P) placebo, (C) corticosterone, (∅) no exogenous corticosterone released. ***$P<0.001$ as determined by Student's t-test relative to appropriate placebo control. Results shown are mean±s.e.m. with $n=6–8$ mice per treatment group.

implanted with corticosterone or placebo pellets plasma corticosterone levels were between 20 and 50 ng/ml for placebo treated animals and between 110 to 150 ng/ml for mice treated for 7, 14 or 21 days with corticosterone (40 mg/kg/day). Mice implanted for 28 days (i.e. with a 7 day washout period) showed a plasma corticosterone concentration of approximately 50 ng/ml i.e. levels had returned to baseline. Statistical analysis of the chronic treatment data showed a significant effect of treatment ($F_{(1,49)}=21.02$, $P=0.00003$) but not of time although there was a significant interaction between treatment and time of treatment ($F_{(3,49)}=3.40$, $P=0.02$). Post hoc analysis using Fisher's LSD test revealed that 7, 14 and 21 day corticosterone treatments resulted in statistically significant increases in plasma corticosterone concentration ($P=0.0004$, 0.026 and 0.0008 respectively) while the 28 day group was not statistically significant from placebo control ($P=0.74$) (Fig. 1).

Adrenal gland weights were significantly affected by both treatment ($F_{(1,52)}=51.7$, $P<0.00001$) and time ($F_{(3,52)}=3.61$, $P=0.019$) and there was a significant interaction between treatment and time ($F_{(3,52)}=3.01$, $P=0.038$). Post hoc analysis using Fisher's LSD test showed that animals implanted for 7, 14 and 21 days with corticosterone pellets (40 mg/kg/day) showed a significant 23% ($P=0.05$), 47% ($P=0.001$) and 53% ($P=0.00001$) reduction in adrenal gland weights respectively as compared to placebo controls while mice implanted for 28 days showed no significant reduction in adrenal gland weight as compared to placebo controls ($P=0.20$), consistent with a lack of corticosterone being released for the last 7 days of implantation resulting in a restoration of the natural secretion of corticosterone from the adrenals (Fig. 1). There was no significant change in body weight in any of the treatment groups (data not shown).

3.2. The effects of corticosterone administration on hippocampal cell proliferation and volume

Quantification of the number of BrdU positive nuclei (Fig. 2) showed no significant effect of 14 day corticosterone treatment

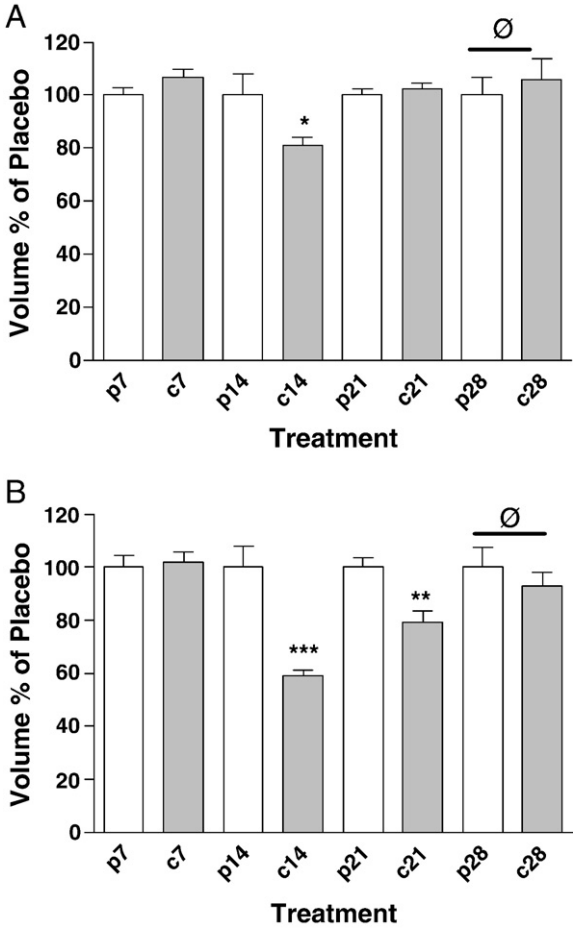

Fig. 3. The effects of chronic corticosterone administration on hippocampal and granule cell layer volume. (A) Hippocampal and (B) granule cell layer volumes were measured using Cavalieri's principle. *$P<0.05$ as determined by Student's t-test relative to placebo control, **$P<0.01$, ***$P<0.001$ as determined by 2 way ANOVA of granule cell layer data with Fisher's LSD post hoc analysis relative to appropriate placebo control (p) placebo (c) corticosterone (∅) no exogenous corticosterone released. Results shown are mean±s.e.m. with $n=6–8$ mice per treatment group. The mean±s.e.m. volumes for placebo treated animals were 11.8±0.4 mm^3 and 0.48±0.02 mm^3 for hippocampus and granule cell layer respectively.

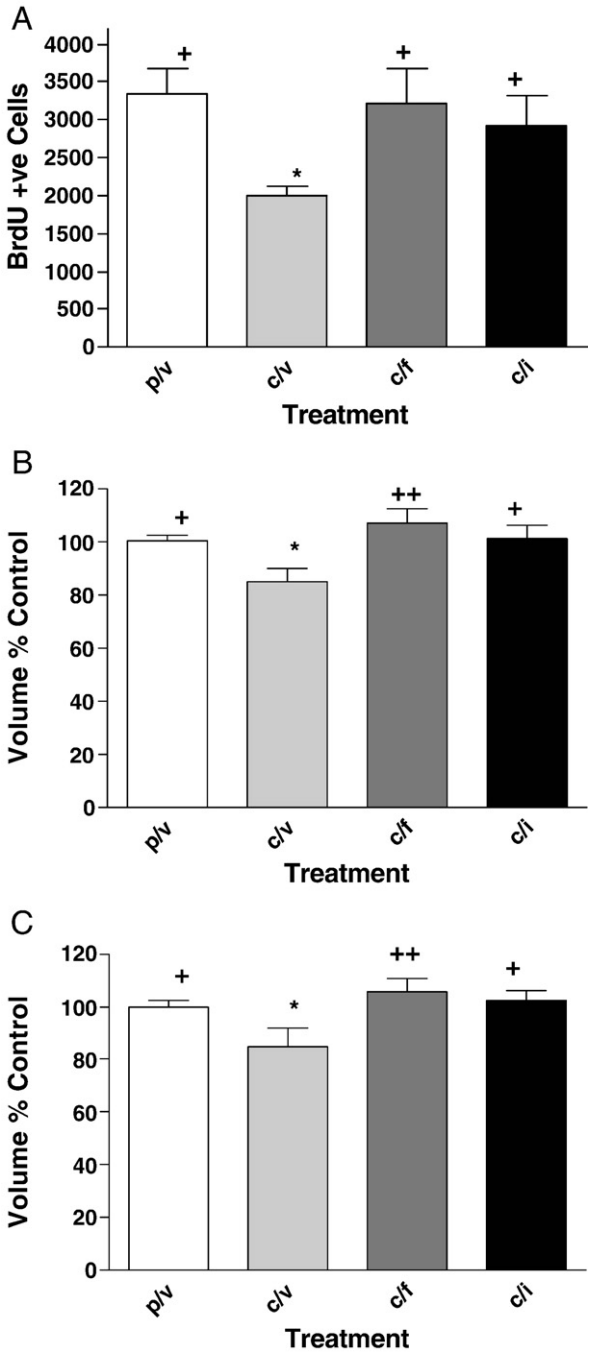

Fig. 4. The effects of 14 day treatment with antidepressants on decreased cell proliferation and hippocampal and granule cell layer Volume Induced by Chronic Corticosterone Administration. (A) Hippocampal cell proliferation was quantified using BrdU immunohistochemistry following 14 day treatment with either saline, fluoxetine (10 mg/kg i.p.) or imipramine (10 mg/kg i.p.) in animals implanted with either placebo or corticosterone (40 mg/kg/day) pellets (B) Hippocampal and (C) granule cell layer volumes were measured using Cavalieri's principle as described in Section 2.4.4. *$P<0.05$ relative to placebo/vehicle, +$P<0.05$, ++$P<0.01$ as compared to corticosterone/vehicle as determined by one way ANOVA with Fisher's LSD post hoc test. (p) placebo, (c) corticosterone, (v) vehicle, (i) imipramine, (f) fluoxetine. * $P<0.05$ relative to placebo/vehicle, +$P<0.05$ relative to corticosterone/vehicle as determined by one way ANOVA with Fisher's LSD post hoc test. Results shown are mean±s.e.m. with $n=6$–8 mice per treatment group.

(10 mg/kg/day) on hippocampal neurogenesis ($P=0.29$, Student's t-test) (data not shown). In a second study to compare 20 and 40 mg/kg/day for 14 days there was a significant effect of corticosterone treatment ($F_{(2,14)}=24.94$, $P=0.00002$) on hippocampal neurogenesis. Post hoc analysis of the data using Fisher's LSD test revealed that while 20 mg/kg/day failed to affect neurogenesis ($P=0.54$) 40 mg/kg/day gave a robust and

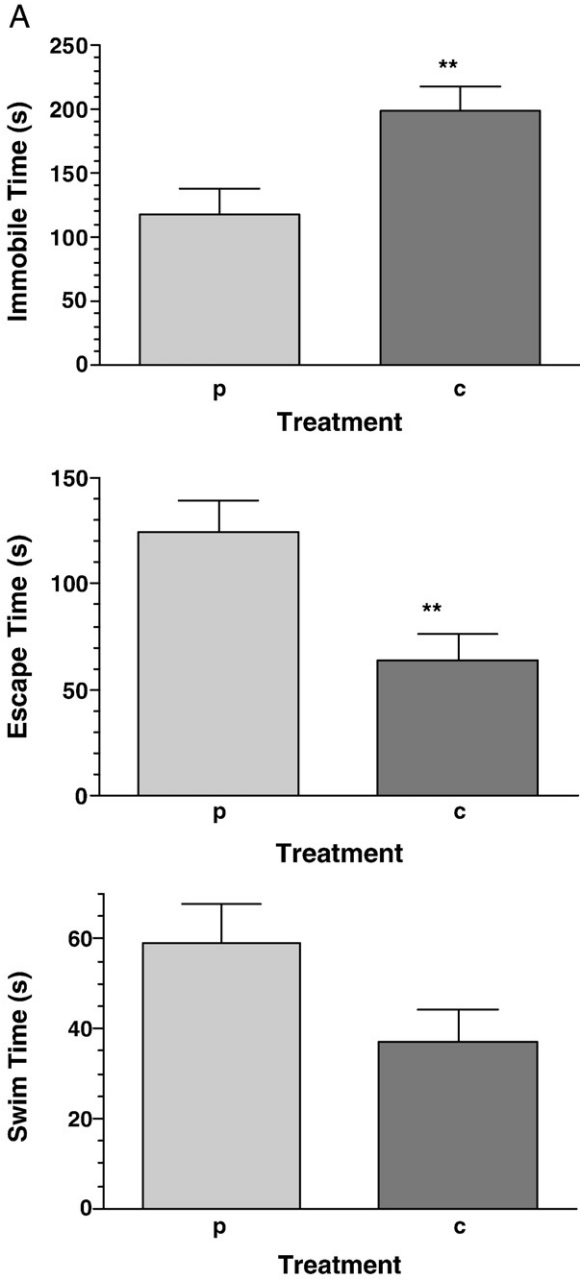

Fig. 5. The effects of corticosterone administration in the forced swim test. Activity in the forced swim test was monitored using videotrack software following (A) acute or (B) chronic administration of corticosterone (40 mg/kg) (P) placebo, (C) corticosterone, (DMI) desipramine (20 mg/kg i.p.). *$P<0.05$, **$P<0.01$ as determined by Student's t-test relative to appropriate vehicle or placebo control. Results shown are mean±s.e.m. with $n=8$ mice per treatment group.

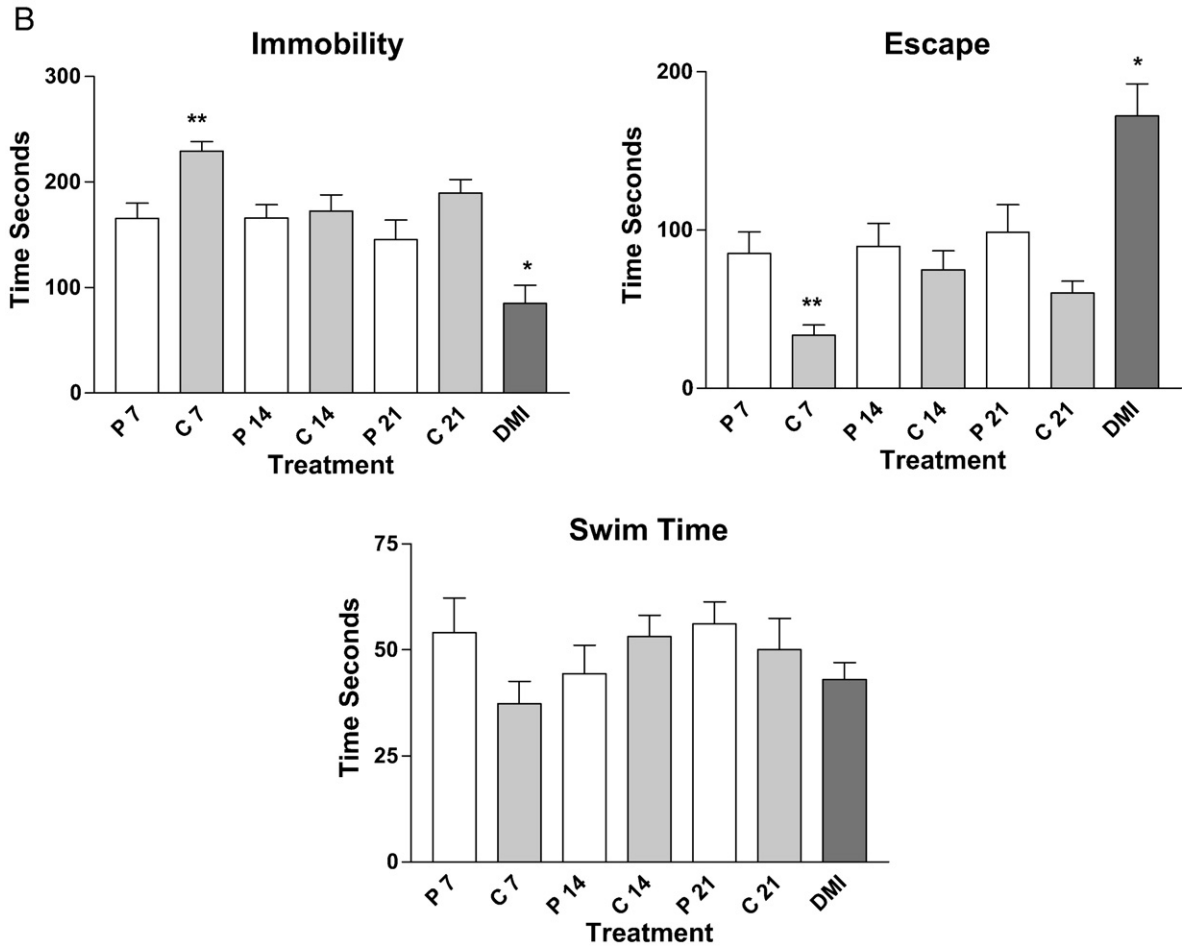

Fig. 5 (*continued*).

significant 50% reduction (mean±s.e.m., 3642±279 positive cells for placebo treated mice versus 1808±88 positive cells for corticosterone treated, $P=0.0002$) of hippocampal neurogenesis (Fig. 2), and therefore this dosing regime was selected for further studies. A time course looking at the number of BrdU positive cells following 7, 14, 21 and 28 days of corticosterone pellet implantation (40 mg/kg/day) revealed that neurogenesis was reduced by approximately 50% at all time points examined (Fig. 2). Statistical analysis of this data revealed a significant effect of both treatment ($F_{(1,49)}=116.31$, $P<0.00001$) and of time ($F_{(3,49)}=4.62$, $P=0.006$) on hippocampal neurogenesis but no significant interaction between treatment and time ($F_{(3,49)}=0.36$, $P=0.78$). Further analysis of the data was carried out for each of the timepoints, not taking into account multiple comparisons, using individual Student's *t*-test analysis and revealed that 7, 14 and 21 days of corticosterone treatment had significantly reduced hippocampal neurogenesis (all $P<0.001$) compared to their time matched placebo controls. The 28 days time point represented an additional 7 days without corticosterone release based on the manufacturers' guarantee of 21 days release formulation and was verified by taking terminal plasma samples at this timepoint for analysis of corticosterone levels. There was no significant effect of an acute dose of corticosterone (40 mg/kg) ($P=0.53$, Student's *t*-test) on the number of BrdU positive cells (vehicle 1328±119, corticosterone 1434±111).

Hippocampus and granule cell layer volume data were treated separately and analysed by two way ANOVA. Hippocampal volume measurements demonstrated there was no significant effect of treatment ($F_{(1,49)}=0.52$, $P=0.82$) or time ($F_{(3,49)}=2.49$, $P=0.07$) and no significant interaction between treatment and time ($F_{(3,49)}=2.49$, $P=0.07$). Further analysis of the individual time point data, not taking into account multiple comparisons, using individual Students *t*-test revealed that at 14 days of corticosterone treatment there was a small though significant reduction in hippocampal volume ($P=0.04$) as compared to placebo treated animals. granule cell layer data was also analysed by two way ANOVA to examine the effects of treatment and time. Analysis confirmed a significant effect of treatment ($F_{(1,49)}=21.59$, $P=0.00003$), time ($F_{(3,49)}=5.97$, $P=0.0015$) and an interaction between treatment and time ($F_{(3,49)}=5.97$, $P=0.0015$). Post hoc analysis using Fisher's LSD test revealed a significant effect of corticosterone at 14 ($P=0.000007$) and 21 ($P=0.004$) days following implantation. At 28 days following pellet implantation there were no significant differences in granule cell layer volume in the mice which had been treated with corticosterone ($P=0.29$) as compared to placebo treated mice. This time point represented

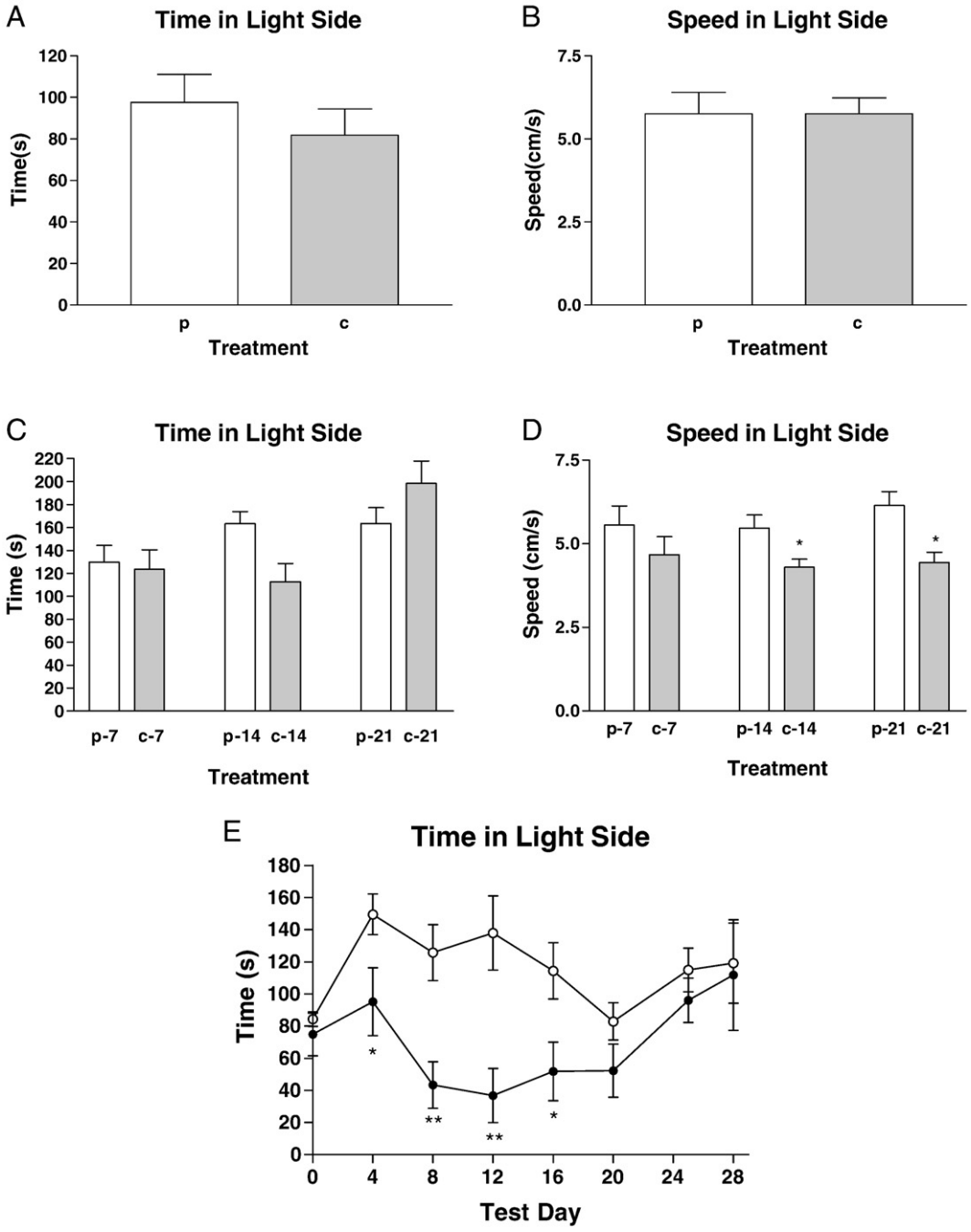

Fig. 6. The effects of corticosterone administration on performance in the light dark box. Performance in the light dark box was measured following acute corticosterone treatment (40 mg/kg i.p.) (A) and (B); weekly following implant of corticosterone pellets (40 mg/kg/day) for 21 days (C) and (D) or every 4 days following implant of corticosterone pellets (40 mg/kg/day) for 28 days (E). (○/p) placebo (●/c) corticosterone. *$P<0.05$, **$P<0.01$ as determined by repeated measures ANOVA with Dunnett's post hoc test. Results shown are mean±s.e.m. with $n=8$ mice per treatment group.

a 7 day wash out and indicated that the normalisation of corticosterone levels had resulted in a return to baseline granule cell layer volume (Fig. 3). There was no significant effect of acute corticosterone treatment on hippocampal or granule cell layer volumes ($P=0.99$ and 0.92 respectively) as determined by Student's *t*-test (hippocampus=99.8±2.5% of vehicle, granule cell layer=99.2±4.5% of vehicle, vehicle volumes were 16.0±0.8 mm^3 and 0.56±0.04 mm^3 for hippocampus and granule cell layer respectively).

3.3. The effects of co-administration of fluoxetine and imipramine on hippocampal cell proliferation and volume

Corticosterone (40 mg/kg/day) treated animals showed a 40% decrease in neurogenesis ($P=0.007$, Fisher's LSD post hoc analysis). Co-administration of imipramine and fluoxetine (both 10 mg/kg i.*p*. for 14 days) prevented the corticosterone induced decrease with 13% and 4% reductions in neurogenesis which were not statistically significant from placebo/vehicle treated

animals ($P=0.32$ and 0.86 respectively, Fisher's LSD post hoc analysis) but were significantly different from corticosterone/vehicle treated animals ($P=0.03$ and 0.05 respectively, Fisher's LSD post hoc analysis) (Fig. 4).

Co-administration of fluoxetine and imipramine with corticosterone showed a significant effect of treatment on both hippocampal ($F_{(3,26)}=4.02$, $P=0.02$) and granule cell layer volumes ($F_{(3,26)}=3.58$, $P=0.02$) as determined by one way ANOVA. Post hoc analysis of the data using Fisher's LSD test illustrated that the hippocampal volume following corticosterone/vehicle treatment was significantly lower (15%) than in placebo/vehicle treated animals ($P=0.03$) (Fig. 4). Co-administration of fluoxetine (10 mg/kg, i.p. for 14 days) or imipramine (10 mg/kg, i.p. for 14 days) prevented this volume deficit ($P=0.002$ and 0.02 respectively as compared to corticosterone/vehicle treated animals, determined by Fisher's LSD post hoc analysis). Similar results were obtained for granule cell layer volumes, again showing a significant decrease (16%) in volume following corticosterone/vehicle treatment ($P=0.03$) which was prevented by 14 days co-administration of fluoxetine ($P=0.006$) or imipramine ($P=0.01$) as determined by Fisher's LSD post hoc analysis.

3.4. The effects of corticosterone administration on performance in the forced swim test

A single acute dose of corticosterone (40 mg/kg i.p.) 1 h before testing in the FST produced a markedly "depressed" phenotype characterised by an increased immobility time ($P=0.009$, Student's *t*-test) which was also accompanied by significantly reduced escape behaviour ($P=0.008$, Student's *t*-test) and a non-significant trend towards decreased swimming behaviour ($P=0.07$, Student's *t*-test) (Fig. 5). Chronic treatment with corticosterone (40 mg/kg/day) revealed a significant effect of treatment on immobility ($F_{(1,39)}=11.3$, $P=0.002$) and escape time ($F_{(1,39)}=10.7$, $P=0.002$) but not on swim time ($F_{(1,39)}=0.79$, $P=0.38$) as determined by two way ANOVA. There were no significant effects of time or any interaction between treatment and time on the three parameters measured. Further analysis of the individual time point data, not taking into account multiple comparisons, using individual Student's *t*-test revealed that at 7 days post corticosterone pellet implant mice showed a "depressed" phenotype with significant changes in immobility ($P=0.002$) and escape ($P=0.004$) behaviours similar to that seen after acute administration (Fig. 5). Interestingly, at 14 and 21 days post pellet implantation, corticosterone animals showed no significant differences in behaviour as compared to placebo controls. The antidepressant desipramine (20 mg/kg i.p. with a 30 min pretreatment time) included as a positive control produced the expected reduction ($P<0.05$) in time spent immobile (Fig. 5).

3.5. The effects of corticosterone on performance in the light dark box

A single 40 mg/kg injection given one hour before testing in the light dark box did not significantly affect time spent in the light side of the box ($P=0.40$, Student's *t*-test) or on speed in the light side of the box ($P=1.00$, Student's *t*-test) as compared to vehicle treated controls (Fig. 6). Mice implanted with corticosterone or placebo pellets and then tested 7, 14 or 21 days after pellet implantation showed no significant effects on performance in the light dark box as a result of treatment ($F_{(1,40)}=0.15$, $P=0.69$) although there was a significant effect of time ($F_{(2,40)}=7.07$, $P=0.002$) with animals at 21 days spending more time in the light side of the box as compared to 7 and 14 days (without taking treatment into account) which may have signified habituation to the test. There was no significant interaction between treatment and time ($F_{(2,40)}=2.82$, $P=0.07$). In contrast, animals which were tested in the light dark box every 4 days showed a very different pattern of behaviour. Pre-implantation testing revealed that both groups of animals displayed similar amounts of time spent in the light side of the box, however at 4, 8, 12 and 16 days post implantation the corticosterone treated mice spent a significantly shorter time in the light side of the box ($P=0.04$, 0.004, 0.006 and 0.03 respectively as determined by Dunnett's post hoc analysis following repeated measures ANOVA). This appeared to be due to the placebo treated mice spending more time in the light side compared to day 0 and the corticosterone animals spending the same or slightly less time in the light side as compared to day 0. Interestingly, at days 24 and 28 there were no significant effects of corticosterone on performance in the light dark box with the two groups spending a very similar amount of time spent in the light side of the box. These represent time points where no exogenous corticosterone was present and plasma had returned to physiological levels as the pellets were 21 days release formulation (Fig. 6).

4. Discussion

Subcutaneous corticosterone pellet implantation caused a moderate, robust and constant elevation of plasma corticosterone in mice increasing levels by approximately 2–3 fold above controls and was maintained for 21 days. Analysis of plasma samples from mice 28 days after pellet implantation confirmed that at this time point plasma levels had returned to baseline levels. These changes in plasma corticosterone levels were mirrored by changes in adrenal gland weights indicating anticipated compensatory changes of endogenous corticosterone production.

Following administration of corticosterone at a dose of 20 mg/kg/day for 14 days there were no effects on cell proliferation however at 40 mg/kg/day there was a marked decrease of around 50% similar to that reported in the literature (Hellsten et al., 2002; Karishma and Herbert, 2002). The lower dose showed no effects on neurogenesis despite having been shown to elicit effects on other biochemical and behavioural paradigms indicative of a "depressed-like" phenotype (Dachir et al., 1995; Marinelli et al., 1997; Brotto et al., 2001). Plasma corticosterone concentrations obtained for the acute injections compared favourably with published values for similar dosing regimes (Inoue and Koyama, 1996) and were approximately 10× higher that those obtained in animals with subcutaneously implanted corticosterone pellets.

Allowing animals to survive for 28 days after implantation permitted the examination of how the HPA axis adapted to withdrawal of exogenous corticosterone. While at 28 days there is clearly no exogenous corticosterone being released from the

pellets, adrenal gland weights were normal (indicating the endogenous production of corticosterone had resumed) and circulating plasma corticosterone levels had returned to baseline however there was still a significant (54%) reduction in hippocampal cell proliferation at this time point. This implies that even when plasma corticosterone levels are normalised hippocampal cell birth is still inhibited and may take more than 7 days to recover. There is some evidence to show that chronic stress paradigms can result in marked upregulation of both cytosolic and nuclear glucocorticoid receptors and that nuclear glucocorticoid receptor upregulation persists for more than 10 days following cessation of chronic stress (Mizoguchi et al., 2003). This may explain the apparent disconnect between plasma corticosterone and hippocampal neurogenesis 7 days following the cessation of corticosterone from the implanted pellets. It may be that for hippocampal neurogenesis to return to baseline levels normal hippocampal glucocorticoid receptor tone must be restored. This could be confirmed by extending the washout period post corticosterone release and correlating neurogenesis with hippocampal glucocorticoid receptor level. There is of course also the possibility, that the reduction in hippocampal neurogenesis mentioned above is irreversible following such chronic glucocorticoid treatment.

The changes of granule cell layer volume did not follow the time course of cell proliferation changes as one might expect if the granule cell layer volume reduction was due to a decrease in newborn cells. It may be that the reductions in granule cell layer volume following corticosterone treatment are indicative of dendritic pruning which has been reported following chronic exposure to glucocorticoids (Wellman, 2001), although this seems unlikely due to the relatively small number of dendrites in the granule cell layer (Seki et al., 2007). The reduction in hippocampal volume seen in depression may not be due to hippocampal damage via apoptosis (although apoptosis was demonstrated) or dendritic pruning but was in fact due to a redistribution of tissue water balance in the affected area (Lucassen et al., 2001) this theory is also supported by studies showing that alterations in HPA axis activity which elevate glucocorticoids affects water balance via a hypothalamic mechanism (Kellner et al., 1995). This may explain the temporal disconnect between granule cell layer neurogenesis effects and granule cell layer/hippocampal volumes since the work by Mizoguchi et al. (2003) shows that changes in hypothalamic glucocorticoid receptors following chronic stress are much less marked and return closer to baseline values on withdrawal of stressors than in hippocampus.

Long term treatment with both fluoxetine and imipramine prevented the corticosterone induced decrease in cell proliferation and hippocampal and granule cell layer volumes. While of interest, the mechanism behind the prevention of the corticosterone deficit is unclear. Thus, it may be that antidepressants act to normalise glucocorticoid balance, an effect suggested to be due to their influence on membrane steroid transporters resulting in increased negative feedback (Pariante et al., 1997) although in this case it would be difficult to reconcile this argument due to the continuously elevated levels of exogenous corticosterone released in this paradigm. The large reduction in adrenal gland weight would also imply that endogenous corticosterone production had all but ceased thus minimising any influence on the HPA axis. It may therefore be more likely that antidepressants counteract the glucocorticoid effects by increasing the birth of new cells or decreasing the death of these newborn cells by glucocorticoid independent mechanisms by for instance increasing the amount of growth factors present in the hippocampus or by influencing pro and anti-apoptotic factors (Murray and Hutson, 2007). It is not possible to exclude other possible mechanisms that chronic antidepressant treatment may induce as was shown for the interaction between chronic stress and antidepressant treatment (Raone et al., 2007).

The results from the forced swim studies show that both acute injection and 7 day pellet implantation resulted in a “depressed” phenotype as demonstrated by an increased immobility time and reduced escape behaviour. Animals treated for 14 and 21 days however showed no change in this behaviour. It is unlikely that this is an artefact due to changes of locomotor activity as chronic corticosterone treatment moderately reduced locomotor activity (data not shown) which might be expected to be manifest as a “depressed” phenotype at the later but not earlier time points in the forced swim test, which is clearly not the case. Results imply that an adaptive change has occurred following chronic corticosterone treatment which has resulted in a loss of the “depressed” phenotype seen at the earlier two time points. The fact that all animals were only exposed to the assay once eliminates the possibility that habituation to the test has occurred. Any influence of corticosterone on locomotor activity can also be discounted as the 1 h pretreatment in the case of the acute test was before any locomotor effects were observed. Furthermore, since the effect of acute corticosterone administration was to increase locomotor activity (data not shown) it would be expected that the corticosterone treated animals would exhibit an “antidepressant” like phenotype rather than the “depressed” phenotype that was clearly observed. Similarly locomotor effects are unlikely to confound the 14 and 21 days results as there was a tendency to decrease locomotor activity at 21 days which would only serve to enhance a depressed behaviour by falsely increasing immobility time and reducing swim and escape behaviour. Additionally, it is improbable that the effects of acute and 7 day corticosterone in the forced swim test are purely due to circulating levels of corticosterone as the plasma levels of the hormone remain elevated in implanted animals at 7, 14 and 21 days. Taken together these data endorse the possibility of an adaptive change following exposure to corticosterone for greater than 7 days and of repeated exposure to stress (which would be accompanied by elevated corticosterone) (see Ni et al., 1999; Schaaf et al., 2000).

Perhaps most relevant to the forced swim test data in the present study is that chronic (10 days) stress, and presumably concomitantly elevated corticosterone, also increased hippocampal TrkB mRNA expression (Nibuya et al., 1999) a phenomenon which does not occur following acute stress. This is worthy of note because it has been shown that mice which over-express a truncated (and therefore inactive) form of TrkB are non responders to antidepressants in the forced swim test (Saarelainen et al., 2003) leading the authors to suggest a crucial

role for TrkB and its ligand BDNF in the forced swim test. It may therefore be that the "depressed" phenotype seen with acute and 7 day antidepressant treatment is attenuated by an upregulation of TrkB receptors (thought to be a compensation for decreased levels of BDNF) resulting in an increase in antidepresssant like behaviour which masks the initially "depressed" phenotype seen at the early time points, such effects of corticosterone on BDNF and TrkB have been reported in the literature (Schaaf et al., 1997). Furthermore recent work from Kozlovsky et al. (2007) are supportive of this hypothesis as they show that chronic stress (which resulted in elevated corticosterone markedly decreased mRNA for BDNF with corresponding increase in mRNA for TrkB. Another possibility may be that the effects in the forced swim test are under the influence of glucocorticoid receptors and that following chronic administration of corticosterone glucocorticoid receptors are downregulated, an occurrence which has previously been reported both *in vivo* and *in vitro* (Erdeljan et al., 2001; Bisagno et al., 2000), resulting in a loss of "depressed" phenotype in this assay. This may be as a direct result of reduced glucocorticoid receptor related gene transcription an event that has been shown to be blocked by antidepressant administration (Budziszewska et al., 2000). It should not be forgotten however that the forced swim test is a behavioural test specifically designed to identify antidepressant activity and not to identify a "depressed" phenotype. Therefore the present studies to detect "depressed" behaviours may be at the limit of detection or indeed may be an inappropriate interpretation of the data obtained.

The effects seen in the forced swim test are quite different to those observed in the light dark box which seem to manifest with time (or repeated exposure to the test arena) rather than diminish as seen in the forced swim test. Whilst the light dark box test has traditionally been used to measure anxiolytic activity (Crawley, 1981) it has also been shown to be sensitive to chronic (Keeney and Hogg, 1999) but not acute antidepressant treatment (Bourin and Hascoet, 2003). Initial studies using the light dark box showed no significant effect of elevated glucocorticoid levels at any of the time points tested, which probably suggests that purely elevating corticosterone levels is not sufficient to elicit a response in this paradigm. It was subsequently decided to modify the testing protocol to better reflect the paradigm used by Keeney and Hogg (1999) by testing before corticosterone implant and then testing every 4 days for the duration of exogenous corticosterone release and then a further two time points following the cessation of exogenous corticosterone release representing a 7 day washout period.

The results from this paradigm showed markedly different effects to the weekly testing paradigm with placebo and corticosterone treated mice showing a similar level of exploratory activity in both compartments prior to corticosterone implantation but a clear "anxious/depressed" phenotype emerging with time in the corticosterone treated animals. However, further examination of the data revealed that the result may not be a straightforward change in anxiety state. The placebo treated animals appear to increase the amount of time spent in the light side of the box (presumably representing habituation following repeated exposure to the box) suggesting that they gradually perceive the light side of the box is less aversive than on first exposure. This adaptation to the light dark box has been previously reported (Onaivi and Martin, 1989) although different strains of mice were used (a variable which has been purported to be a factor impacting performance in this test (Bourin and Hascoet, 2003). Unlike the placebo treated animals the corticosterone pellet implanted animals did not show an increase in time spent in the light side of the box on the fourth day of treatment and showed a decrease in the amount of time spent in the light side between days 4 and 20. Therefore the significance of corticosterone treatment and time in this paradigm is partly due to less time spent in the light side by the corticosterone treated animals but is also due to the increased time spent in the light side by the placebo treated animals. On days 24 and 28 of testing the increase of time spent in the light side of the box coincided with the cessation of corticosterone release by the 21 day pellets suggesting that the increased anxiety/lack of habituation in these mice is dependent on elevated circulating corticosterone and is normalised on withdrawal of exogenous glucocorticoids.

These data suggest there may be a cognitive element involved in the performance of the placebo treated animals in this paradigm and which may be reduced or absent in the corticosterone treated animals. Given the extensive reports of an involvement of glucocorticoids and glucocorticoid receptors in cognitive processing in both man and laboratory animals (Arbel et al., 1994; Newcomer et al., 1994) this suggestion seems plausible and is supported by the fact that a lack of habituation to the light dark box seems to track plasma corticosterone levels.

Taken together results in the present studies indicate that chronic exposure to moderately elevated corticosterone was sufficient to decrease hippocampal neurogenesis and granule cell layer volume. These changes were prevented by chronic treatment with clinically efficacious antidepressants further strengthening the view that antidepressants may be effective in the treatment of depression by counteracting the cellular changes induced by the effects of prolonged stress.

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