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# Neuroprotection in steroid therapy: a rodent model

## Key Messages

1. Chronic steroid therapy causes disturbance in cell proliferation of the hippocampus and the subventricular zone. This may be the underlying cause of altered memory and cognitive function.
2. Co-administration of paroxetine (a class of antidepressants) during steroid therapy could counteract the destruction. Modification of the current steroid therapy regimen may be required.

## Introduction

Corticosteroid decreases neural cell production in the hippocampus,<sup>1</sup> whereas antidepressants induce neurogenesis.<sup>2</sup> The hippocampus is a brain region for memory formation. Decreased production of neurons in this region has a negative impact on cognitive function. We assessed the hypothesis that the neuro-damaging effect of high-dose corticosteroid on the hippocampus and subventricular zone (SVZ) could be reversed by administration of paroxetine—a selective serotonin reuptake inhibitor for treatment of depressive disorders. A rodent model was used to test the effect of paroxetine, corticosterone, and co-treatment of these two drugs on neurogenesis of the hippocampus and SVZ. In patients receiving steroid therapy, the neuroprotective effect of paroxetine suggests that administration of antidepressant could prevent deterioration of neuron production. This study aimed to investigate drug interactions of paroxetine, lithium, and corticosterone on the hippocampus and SVZ in terms of cell proliferation, dendritic morphology, neuronal survival, and molecular mechanisms, and whether selective serotonin reuptake inhibitor and lithium could exert protection against corticosterone-induced neuron damage on the hippocampus and SVZ.

## Methods

This study was conducted from January 2005 to December 2006. Six groups of rats (n=4-5 for each group) were divided into: (1) 14 days of corticosterone injections (40 mg/kg, subcutaneously), (2) 14 days of paroxetine injections (10 mg/kg, intraperitoneally), (3) 14 days of both corticosterone and paroxetine injections (same dosage as above), and (4) 14 days of vehicle injections.

To study the effect of lithium and corticosterone on dendritic morphology, the rats were divided into: (1) 14 days of lithium treatment (85 µg/kg, n=6), and (2) 14 days of lithium and corticosterone treatment (same dosage as above, n=6).

Proliferative cells in the hippocampus and SVZ were labelled by bromodeoxyuridine (BrdU) injection (50 mg/kg) during the final 3 days of treatment. After 14 days of treatment, rats were sacrificed by decapitation, and their brains were processed for BrdU immunohistochemistry or Golgi staining. During the treatment period, another set of experimental rats was sacrificed and their hippocampi were dissected for quantitative polymerase chain reaction.

For the differentiation and neurogenesis study, the rats were divided into 4 groups as described above, with each group containing 6 rats. The rats were allowed to survive for 3 weeks after completion of the 14 days' treatment. The total number of rats used was 135.

### *BrdU immunohistochemistry*

Frozen slices of the hippocampus and SVZ were slide-mounted and boiled in citric acid (pH=6.0) for 10 mins, followed by PBS rinses. The brain sections were incubated in 1M HCl (37°C, 30 mins) and then boric acid buffer (pH=8.5, 10 mins). After blocking with 5% normal goat serum in 0.01% Triton X-100, sections were incubated overnight with anti-mouse BrdU (1:400, Roche) at 4°C. Sections were then incubated for 1 hr with secondary antibody (biotinylated goat

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anti-mouse; Vector Laboratories), followed by amplification with an avidin-biotin complex (Vector Laboratories). Cells were then visualised with diaminobenzidine. For the neurogenesis study, co-immunostaining with rat anti-BrdU antibody (1:1000, abcam) and mouse anti-NeuN (1:1000, Chemicon) were used as primary antibody. Secondary antibodies were goat anti-mouse and rat (Alexor fluor 488 and 563, Molecular Probes). The chemical supplier was Sigma-Aldrich unless otherwise indicated.

### ***Golgi staining***

Golgi staining was carried out using the FD Rapid GolgiStain Kit according to the manufacturer's protocol. In brief, the brains of the treated rats were immersed in the impregnation solution for 2 weeks, after which the tissue was cut into 50-micron-thick sections and stained.

### ***Quantitative polymerase chain reaction***

Hippocampi of the rats in the three treatment groups (corticosterone, paroxetine, and co-treatment) were taken out at different time points: 4 hours, 2 days, 4 days, 7 days, and 14 days after treatment. Hippocampal tissues were dissected and stored at  $-70^{\circ}\text{C}$  until use. Hippocampal RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol. Gene expression of brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB) among different groups of rats at different time points were measured by the iCycler iQ™ Multi-Color RT-PCR System. The primer sequences and probe for amplification of BDNF were: 5'-CTGACACTTTTGAGCACGTGATC-3' (forward), 5'-CGTTGGGCCGAACCTTCT-3' (reverse), and 5'-CATCCAGCAGCTCTTC-3' (probe). The primer sequences of GAPDH (as internal controls) were: 5'-CAGAACATCATCCCTGCATCCA-3' (forward), 5'-CCGTTTCAGCTCTGGGATGAC-3' (reverse), and 5'-CTTGCCACAGCCTTG-3' (probe).

### ***Data quantification and statistical analysis***

BrdU-positive cells on every 12th unilateral section through the whole dentate gyrus were counted at  $\times 1000$  magnification, with the aid of the Stereo Investigator software. BrdU-positive cells in the dentate gyrus were counted from 2400 to 3600  $\mu\text{m}$  posterior to Bregma. In SVZ, BrdU cells were counted from 1800 to 0  $\mu\text{m}$  anterior to Bregma. The number of BrdU-labelled cells per dentate gyrus was then multiplied by 24 to estimate the total number of BrdU-positive cells through the dentate gyrus. The number of BrdU-positive cells in the hippocampal subgranular zone in each group was defined as a percentage of control (vehicle). For SVZ sections, the cell number was expressed as the number of cells per section.

### ***Brain-derived neurotrophic factor enzyme-linked immunosorbent assay***

BDNF enzyme-linked immunosorbent assay (ELISA) kits (Chemicon) were used to assess hippocampal BDNF protein level after 14 days of treatment. Protein extracts

were prepared from snap-frozen rat hippocampi. For each rat, 50  $\mu\text{g}$  of protein extract was used for assay. ELISA was carried out according to the manufacturer's protocol.

## **Results**

### ***Adrenal gland atrophy caused by subchronic corticosterone treatment***

After 14 days of treatment, the adrenal glands were dissected and weighed. Respectively in the control, corticosterone, co-treatment, and paroxetine groups, the mean adrenal weights (in g) were  $27.63 \pm 4.85$ ,  $6.20 \pm 2.78$ ,  $10.633 \pm 1.46$ , and  $45.47 \pm 14.15$ . Adrenal weights of the rats in the corticosterone and co-treatment groups decreased, which indicated hypercortisolaemia induction and chemically induced lesions. This confirmed that the rats were subjected to chronic exposure of high-dose corticosterone during the treatment period.

### ***Hippocampal neurogenesis after corticosterone and paroxetine treatment***

In the cell proliferation assay, the number of proliferating cells was identified by immunohistochemical detection of BrdU within the nuclei of actively dividing cells. The BrdU-positive nuclei were clustered in the subgranular layer and hilus and exhibited irregular shape. In the experiment, only subgranular layer cells were counted. Compared to the controls, corticosterone significantly decreased the number of BrdU-labelled cells in the dentate gyrus ( $2470.00 \pm 31.56$  vs  $1677.38 \pm 146.97$ ,  $P < 0.05$ ). Chronic treatment with paroxetine significantly increased BrdU-labelled cells ( $13255.71 \pm 83.98$ ,  $P < 0.001$ ) in the subgranular and granule cell layer, compared to the corticosterone or vehicle-alone group. The number of BrdU-positive cells in the co-treatment group ( $2204.48 \pm 90.40$ ) was not significantly different from the vehicle-treated controls. For neuronal differentiation, no difference in the percentage of BrdU-labelled cells showing NeuN expression was noted among the four groups. This indicated that subchronic corticosterone treatment decreased hippocampal neurogenesis, whereas co-treatment with paroxetine increased the effect to a level similar to controls. In short, antidepressant therapy may be effective in preventing neurological damage caused by subchronic or chronic steroid therapy.

### ***Morphological analysis by Golgi staining***

Brain tissues were subjected to Golgi staining to study morphological changes (dendritic trees and spines) of hippocampal neurons after treatment. In the corticosterone, lithium, and co-treatment groups, at least five neurons were traced using the NeuroLucida software, and the data were analysed using the NeuroExplorer software. The number of neuronal nodes (ie the number of dendritic tree branches) in the corticosterone group was significantly lower than in the lithium and co-treatment groups ( $13.5 \pm 4.36$  vs  $32 \pm 6.00$  vs  $33.63 \pm 12.00$ ,  $P < 0.05$ ). The mean dendritic length of each neuron (in  $\mu\text{m}$ ) in the corticosterone group was significantly smaller than in the other two groups ( $410.0 \pm 49.7$  vs

1762.6±182.4 vs 2068.6±782.9,  $P<0.05$ ). This indicated that corticosterone decreased dendritic length and the number of dendrite branches, whereas corticosterone plus lithium could reverse the changes.

#### ***Effect of drug treatment on hippocampal cell death***

Cell death in the hippocampus was assessed by Nissl staining. No cells with pyknotic appearance (ie undergoing apoptotic cell death) were detected with cresyl-violet staining in any treatment group. This indicated that subchronic corticosteroid treatment was unlikely to cause neurological damage by increasing hippocampal cell death, and that antidepressants may not exert their therapeutic effect by protecting neurons from apoptosis.

#### ***Quantification of change of brain-derived neurotrophic factor and cAMP response to element-binding protein after drug treatments***

The quantitative polymerase chain reaction was used for detecting alteration of BDNF and CREB levels in the hippocampus. In contrast to expectation, BDNF gene expression levels in all treatment groups decreased but not significantly across the treatment period when compared to day 0.

#### ***Brain-derived neurotrophic factor protein expression by enzyme-linked immunosorbent assay***

The BDNF level in the hippocampus decreased significantly in corticosterone-treated rats than in vehicle-treated rats (66.88±4.22 vs 90.2±6.63 ng/mL,  $P<0.05$ , ANOVA with LSD post-hoc test). Co-treatment with corticosterone and paroxetine (87.73±6.65 ng/mL,  $P>0.05$ ) prevented the effect of decreased BDNF expression, compared to controls. No significant difference was noted between the vehicle group and paroxetine-treated group (90.2±6.63 vs 99.07±8.42 ng/mL,  $P>0.05$ ).

#### ***Effect of corticosteroid and paroxetine on subventricular zone neurogenesis***

Similar to the findings for hippocampus cell proliferation, corticosterone treatment significantly reduced the number of BrdU-positive cells in SVZ, whereas paroxetine treatment significantly increased the number. Respectively in the control, corticosterone, paroxetine, and co-treatment groups, the numbers of BrdU-positive cells in SVZ were 324.0±33.3, 264.1±28.1, 434.9±36.2, and 330.9±23.2 cells/section ( $P<0.05$  for control vs corticosterone and control vs paroxetine).

### **Discussion**

Using the cell birth-dating technique (BrdU labelling), the numbers of proliferative cells in the hippocampus and SVZ were noted to increase with paroxetine treatment. However, no pyknotic cells were observed in the hippocampus in any of the treatment groups, indicating that the drugs had no significant effect on cell survival in our treatment paradigm. The neurogenesis-promoting effect of antidepressants may be due to their influence on the serotonergic system and

thus the serotonergic pathways.<sup>3</sup> Lithium could reverse the adverse effect of corticosterone on the dendritic complexity of the hippocampal CA3 region. Administration of lithium during steroid therapy may prevent the undesirable effect of high-dose steroid, but further investigation is needed to determine its behavioural consequence. The intracellular mechanisms responsible for the neurogenic effect remain unclear, but are likely to involve more than one intracellular pathway. Previous studies have suggested a major role of the cAMP-CREB cascade<sup>4</sup> in the process, and that BDNF may be essential for neurogenesis. In our study, the ELISA data showed increased levels of BDNF in paroxetine-treated rats.

### **Conclusions**

Paroxetine, a potent selective serotonin reuptake inhibitor, could reverse the adverse effect of corticosteroid on hippocampal and SVZ neurons. Paroxetine could induce cell proliferation in both neurogenic regions and restore the number of hippocampal proliferative cells in corticosteroid-treated rats. Dendritic morphology study revealed that lithium may be beneficial for dendritic arborisation under the stress condition created by corticosterone treatment. Future studies may investigate the behavioural consequence of altered neurogenesis and dendritic morphology. Also, the molecular interaction between BDNF and CREB is worth studying. Understanding the molecular mechanisms in the neuroprotection of antidepressants may help patients undergoing steroid therapy in preventing cognitive deterioration.

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1. Qiu G, Helmeste DM, Lau WM, Lee TM, Tang SW, So KF. Modulation by paroxetine of suppressive effect of corticosterone on adult hippocampal cell proliferation. *Neurosci Bull* 2007;23:3.
2. Lau WM, Qiu G, Helmeste DM, Lee TM, Tang SW, So KF. Corticosteroid decreases subventricular zone cell proliferation, which could be reversed by paroxetine. *Restor neurol neurosci* 2007;25:17-23.

### **References**

1. Hellsten J, Wennstrom M, Mohapel P, Ekdahl CT, Bengzon J, Tingstrom A. Electroconvulsive seizures increase hippocampal neurogenesis after chronic corticosterone treatment. *Eur J Neurosci* 2002;16:283-90.
2. Malberg JE, Eisch AJ, Nestler EJ, Duman RS. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* 2000;20:9104-10.

3. Jacobs BL, Tanapat P, Reeves AJ, Gould E. Serotonin stimulates the production of new hippocampal granule neurons via the 5-HT1A receptor in the adult rat. *Soc Neurosci Abstr* 1998;24:199.
4. Coppel AL, Pei Q, Zetterstrom TS. Bi-phasic change in BDNF gene expression following antidepressant drug treatment. *Neuropharmacology* 2003;44:903-10.