Eszopiclone and fluoxetine enhance the survival of newborn neurons in the adult rat hippocampus

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Abstract
Clinical research has shown that co-administration of eszopiclone, a sedative-hypnotic sleeping agent, and fluoxetine, a serotonin uptake inhibitor, exerts an additive antidepressant action in treating patients with both depression and insomnia. Preclinical studies demonstrate that the behavioural actions of antidepressants are linked to neurogenesis in the adult hippocampus. To test the hypothesis that the additive effects of eszopiclone and fluoxetine could act via such a mechanism, the influence of combined administration of these agents on the proliferation and survival of bromodeoxyuridine (BrdU)-labelled newborn cells in the hippocampus of adult rats was determined. Chronic eszopiclone + fluoxetine co-administration significantly increased the survival, but not proliferation, of newborn neurons in dorsal hippocampus by approximately 50%, an effect greater than either drug alone. These findings are consistent with the hypothesis that eszopiclone enhances the antidepressant action of fluoxetine, in part via a novel mechanism that increases the survival of newborn neurons.

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Introduction
Major depressive disorder (MDD) is a severe, potentially fatal illness which currently ranks among the top ten causes of disability in the world’s population (Lopez et al. 2006) and is projected to rank among the top three causes of disability by 2020–2030 (Mathers & Loncar, 2006). Currently available chemical antidepressant therapies have limited efficacy, with only 65% of people with depression displaying measurable improvement after any of several antidepressant medications (Nestler et al. 2002). Depression is also often comorbid with other disorders, notably insomnia, which is one of the diagnostic criteria of MDD and is reported in more than 90% of depressed patients (Ford & Kamerow, 1989; Thase, 1999). Therapies which prove efficacious in managing depressive symptoms as well as treating insomnia would therefore be beneficial in the treatment of the subset of patients suffering from a combination of these two disorders.

Clinical studies have shown that co-administration with fluoxetine, a classical selective serotonin reuptake inhibitor (SSRI), and eszopiclone, a non-benzodiazepine sedative-hypnotic agent used to treat insomnia (Najib, 2006), is effective in treating cases where depression and insomnia are comorbid (Fava et al. 2006). The latter study assessed patients meeting DSM-IV criteria for both MDD and insomnia and found that subjects receiving both fluoxetine and eszopiclone had significantly greater changes in clinical assessments of depression [scores on the 17-item Hamilton Rating Scale for Depression (HAMD17), Clinical Global Impression – Improvement (CGI-I), and Severity (CGI-S) items, and greater number of responders and remitters] as well as significant improvements in insomnia symptoms (decreased sleep latency, decreased wake time after sleep onset (WASO),
increased total sleep time (TST), increased sleep quality, and increased depth of sleep). Importantly, improvement in measures of depression was significantly higher in the co-administration group compared to the fluoxetine alone group, suggesting that the combination exerted a synergistic antidepressant effect. Investigating the mechanisms underlying this effect will therefore enrich the understanding of depression, antidepressant treatment, and sleep disorders associated with depression.

Adult hippocampal cell proliferation and neurogenesis, which involves the proliferation of neural progenitors, survival of newly proliferating cells, and differentiation to neural phenotypes, has been implicated in the pathophysiology and treatment of depression (Perera et al. 2008). All classes of antidepressants up-regulate hippocampal neurogenesis (Banasr & Duman, 2007; Duman, 2004), which is required for the behavioural effects of antidepressant treatment in certain animal models of the disorder (Santarelli et al. 2003). Furthermore, depression and antidepressant treatment may alter levels of neurotrophic factors known to regulate adult hippocampal cell proliferation (Duman, 2004).

The present study was conducted to determine if eszopiclone administration promotes new cell birth and survival in the dentate gyrus (DG) of adult rat brain, and if fluoxetine+eszopiclone co-administration has an additive effect under the same measures. We analysed the effects of eszopiclone, whether alone or co-administered with fluoxetine, on cell proliferation and survival in adult rat hippocampus, as well as the phenotype of surviving cells.

**Methods**

**Animals**

Adult male Sprague–Dawley rats (Charles River Laboratories, USA) initially aged 8 wk and weighing 230–250 g were used for the experiments. Animals were housed, two per cage, under standard laboratory conditions (12-h light/dark cycle, lights on 07:00 hours) and constant temperature (25°C) with ad libitum access to food and water. All procedures were in accordance with National Institutes of Health guidelines for animal research and approved by the Yale Animal Care and Use Committee.

**Drug treatment**

For the proliferation study, rats (n = 6 per group) were administered a combination of water and eszopiclone (10 mg/kg, dissolved in 50 mM acetate buffer and adjusted to pH 4.5 with glacial acetic acid to improve solubility), acetate buffer and fluoxetine (5 mg/kg, dissolved in water), eszopiclone (10 mg/kg) and fluoxetine (5 mg/kg), or water and acetate buffer by intraperitoneal (i.p.) injection at 08:00 hours for 21 d. The dosing schedules are based on previous studies of fluoxetine on neurogenesis (Kodama et al. 2004; Malberg et al. 2000; Nakagawa et al. 2002) and of eszopiclone on behaviour (Carlson et al. 2001; Xi & Chase, 2008). A pH of 4.5 was used for the vehicle in order to dissolve eszopiclone. For the survival study, rats (n = 6 per group) were first administered bromodeoxyuridine (BrdU; Sigma, USA) (detailed below) and then administered drugs in exactly the same combinations and dosages for 28 d. The survival study was repeated (n = 6 per group) so that the final number of animals when both experiments were combined was 12 per group. Some animals/samples were excluded for the following reasons: there were three animals in which the levels of BrdU incorporation were low or undetectable (one control, one fluoxetine, and one fluoxetine+eszopiclone), one animal from the fluoxetine group was excluded because it became sick, one control animal was excluded because of high background after immunohistochemical staining, one additional animal from the control group was excluded because it was more than 2 standard deviations away from the mean.

Drug injection volumes were calculated according to base weight and equalled 1 ml/kg body weight per drug. Eszopiclone was obtained from Sepracor (USA), and fluoxetine was obtained from Toronto Chemical Inc. (Toronto, Canada).

**BrdU administration**

For the proliferation study rats received two i.p. injections of 150 mg/kg BrdU spaced 2 h apart with the first BrdU injection occurring 30 min after the last drug administration. For the survival study, rats received a single 150 mg/kg BrdU injection per day over 3 d prior to the beginning of drug administration. In some cases (3/72 for both the proliferation and survival studies) the number of BrdU+ cells was very low or undetectable, presumably due to variation in the absorption, uptake or incorporation of BrdU. This explains the different reported final group sizes (see Drug treatment section above). In the proliferation study rats were perfused 24 h after the last BrdU injection and in the survival study animals were perfused 4 h after the last drug injection.
**Immunohistochemistry**

Animals were anaesthetized with 400 mg/kg chloral hydrate and transcardially perfused with 100 ml of 0.1 mol/l cold phosphate-buffered saline (PBS), followed by 200 ml of 4% cold paraformaldehyde. Brains were harvested and post-fixed overnight in 4% paraformaldehyde in PBS at 4 °C and cryoprotected in 30% sucrose. Serial coronal brain sections (40-μm thick) were cut using a freezing microtome, and were stored in glycerol-based cryoprotectant. Free-floating sections were subjected to DNA denaturation by incubation for 2 h in 50% formamide/2× standard saline citrate (SSC) at 65°C and then incubated for 30 min in 2 N HCl, xylene citrate (SSC) at 65°C. Serial coronal brain sections (40-μm thick) were cut using a freezing microtome, and were stored in glycerol-based cryoprotectant. Free-floating sections were subjected to DNA denaturation by incubation for 2 h in 50% formamide/2× standard saline citrate (SSC) at 65°C, followed by a 2× SSC rinse. Sections were then incubated for 30 min in 2 N HCl, PBS, and then for 10 min in 0.1 mol/l boric acid (pH 8.5). Sections were incubated for 20 min in 3% hydrogen peroxide to eliminate endogenous peroxidases followed by 30 min in blocking buffer (3% normal horse serum in 0.3% Triton X-100, PBS). The cells were then incubated with mouse anti-BrdU (1:100; Becton Dickinson, USA) with blocking buffer overnight at 4°C. Sections were incubated for 1 h with biotinylated horse anti-mouse antibody (Vector Laboratories, USA) with blocking buffer, followed by amplification with an avidin-biotin complex (Vector Laboratories), and cells were visualized with diaminobenzadine (Vector Laboratories) with a light microscope (Zeiss Axioskop; Carl Zeiss, Germany).

For co-labelling studies, brain sections were incubated for 30 min with 2 N HCl and PBS, followed by blocking buffer (3% normal goat serum, 0.3% Triton X-100, PBS). Sections were then incubated for 2 d at 4°C with rat anti-BrdU (1:100, Accurate; Harlan Olac, UK) and mouse anti-neuronal nuclei (NeuN; 1:500, Chemicon, USA). The secondary antibodies were Alexa Fluor 488 goat anti-mouse (1:200) and Alexa Fluor 546 goat anti-rat (1:200; Molecular Probes, USA) and were applied for 1 h and visualized with confocal Z-plane sectioning (1-μm steps) was used to analyse 50 DG BrdU+ cells per animal to confirm the co-localization of both BrdU and NeuN.

**Quantification of BrdU+ cells**

An experimenter blinded to the section codes counted BrdU+ cells. To distinguish single cells within clusters, all counts were performed at 400× under a light microscope (Zeiss Axioskop, Carl Zeiss). For cell counting in the DG, every tenth section throughout the whole hippocampus was processed for BrdU immunohistochemistry. A cell was counted as being in the subgranular zone (SGZ) of the DG defined as the two-cell layer located between the granule cell layer and the hilus. Using Stereo Investigator 8 (MBF Bioscience, USA), which allows precise bounding of regions of interest, coronal sections containing SGZ were demarcated into dorsal or ventral hippocampus using the Paxinos and Watson rat atlas (Paxinos & Watson, 1998) to define precise boundaries. Coordinates were chosen with reference to previous literature (Jayatissa et al. 2008) and were set at dorsal hippocampus spanning −3.10 mm to −4.40 mm relative to bregma referring to the dorsoventral coordinates and ventral hippocampus spanning −4.40 mm to −8.82 mm relative to bregma referring to the dorsoventral coordinates. Counts of BrdU+ cells were conducted over the whole hippocampus with distinction made for dorsal or ventral hippocampus.

For co-labelling, slices were analysed on a confocal microscope (Zeiss Axiovert LSM510, Carl Zeiss). Z-plane sectioning (1-μm steps) was used to analyse 50 DG BrdU+ cells per animal to confirm the co-localization of both BrdU and NeuN.

**Statistical analysis**

The differences among groups were analysed using StatView 5 software (SAS Institute, USA) and analysis of variance (ANOVA), followed by Fisher’s protected least significant difference (PLSD); one-way ANOVA was conducted for each experimental dataset and used for follow-up comparisons. Data are presented as mean ± S.E.M. The level of statistical significance was set at p < 0.05.

**Results**

**Chronic administration of eszopiclone does not significantly affect cell proliferation in the hippocampus**

The number of newborn cells was determined by analysis of dividing cells that incorporate BrdU. The drug administration experimental design for the proliferation study is given in Fig. 1a. There was no significant effect of any of the treatments on the number of newborn BrdU+ cells in the DG compared to the control group (Fig. 1b–d). The effect was similar when analysing dorsal (Fig. 1b) (F(3,28) = 2.68, p = 0.137), ventral (Fig. 1c) (F(3,28) = 1.305, p = 0.300), or whole (Fig. 1d) (F(3,28) = 2.103, p = 0.132) SGZ. The number of BrdU+ cells in the fluoxetine group was 24% higher than the control group. Although not significant, this effect was similar to that observed in previous reports (Malberg et al. 2000; Warner-Schmidt & Duman, 2007). Representative bright-field images are shown for the control (Fig. 1e), eszopiclone (Fig. 1f), and fluoxetine (Fig. 1g) groups.
Chronic administration of eszopiclone in combination with fluoxetine significantly increases the survival of new neurons in the dorsal hippocampus

To examine the possibility that co-treatment influences the survival of newborn cells in the hippocampus, cells were first labelled with BrdU and then eszopiclone + fluoxetine was administered (Fig. 2a). The combination of eszopiclone and fluoxetine significantly increased the number of surviving BrdU+ cells in the dorsal granule cell layer compared to the vehicle group (ANOVA: $F_{3,38} = 3.50$, $p = 0.025$) causing a 47% increase (Fisher’s PLSD: $p = 0.004$) (Fig. 2b). Co-administration also caused a significant increase in number of BrdU+ cells compared to the fluoxetine group in this region (32% increase; Fisher’s PLSD: $p = 0.023$), although fluoxetine was not significant when compared to vehicle. In whole hippocampus co-administration induced a 44% increase in survival vs. vehicle, an effect greater than either drug alone, although not statistically significant (Fig. 2d). In ventral hippocampus the numerical increase in surviving BrdU+ cells, although not significant, was greatest in the fluoxetine group (Fig. 2c). Representative bright-field images are shown for vehicle (Fig. 2e) and eszopiclone + fluoxetine co-administration (Fig. 2f) groups. Note that at this time-point BrdU+ cells are localized in the granule cell layer, as opposed to the SGZ when using the proliferation paradigm (Fig. 1e–g), indicative of migration of newborn neurons into the granule cell layer. Double-labelling demonstrates that ~85% of...
Fig. 2. Effects of chronic eszopiclone, fluoxetine, or combination of eszopiclone + fluoxetine on cell survival and phenotype in granule cell layer (GCL). (a) Survival study drug administration paradigm: bromodeoxyuridine (BrdU) was administered on the first three consecutive experimental days, one morning injection each day, followed by 28 d of drug administration and perfusion 4 h after the last drug administration. (b) Chronic eszopiclone (Esz) and fluoxetine (Flx) co-administration increases cell survival in dorsal subgranular zone (SGZ) as measured by number of BrdU+ cells (eszopiclone dosage 10 mg/kg, fluoxetine dosage 5 mg/kg; ANOVA: F_{3,38} = 3.50, p < 0.05; * indicates Fisher’s PLSD: p < 0.05; vehicle, n = 9; eszopiclone, n = 12, fluoxetine, n = 10; eszopiclone + fluoxetine, n = 11). (c) Chronic drug treatment does not affect cell survival in ventral SGZ. (d) Chronic drug treatment does not affect cell survival in whole SGZ. (e, f) Representative images of BrdU+ cells in dorsal dentate gyrus in (e) control and (f) eszopiclone + fluoxetine co-administration groups (200 × magnification, scale bar 100 μm). Inset in (e) shows detail of BrdU+ cells in GCL indicated in black rectangle; BrdU+ cells appeared as single cells in the survival experiment. (g) The number of BrdU+ and NeuN+ cells for each treatment group was determined. The results are expressed as the percent of total BrdU+ cells (mean ± S.E.M.). A total of 50 cells were analysed for each animal (vehicle, n = 6; eszopiclone; n = 6; fluoxetine, n = 5; eszopiclone + fluoxetine, n = 5). (h) Representative confocal image showing double immunohistochemistry for BrdU (red) and the neuronal marker NeuN (green) in the GCL (400 × magnification, scale bar 25 μm).
the BrdU + cells also express NeuN, a neuronal marker, and this proportion does not change with treatment (ANOVA: $F_{3,18}=0.20, p=0.897$) (Fig. 2g,h).

Discussion

The results of this study provide the first evidence that eszopiclone, in the presence of fluoxetine, affects adult hippocampal cell survival. Although neither eszopiclone nor fluoxetine treatment significantly influenced the survival of newly proliferating cells, co-administration induced a significant increase in survival in dorsal hippocampus.

In the adult hippocampus ~60% of newborn cells die and do not reach a mature stage (Cameron & McKay, 2001). Treatments that increase cell survival may therefore have a significant impact on total cell number. We found that ~85% of the BrdU + cells also expressed NeuN, a marker for neurons, similar to results of previous studies (Cameron & McKay, 2001; Kodama et al. 2004; Malberg et al. 2000). There was no effect of treatment on the percentage of BrdU + and NeuN + cells, indicating that there is an increase in the number of neurons in the dorsal hippocampus. In the present study the lack of effect on cell proliferation distinguishes eszopiclone from antidepressant drugs, consistent with the pharmacological specificity of these agents. Conversely, the ability of eszopiclone to enhance survival in the presence of fluoxetine demonstrates overlap between these agents on this aspect of neurogenesis, as antidepressants are also reported to increase survival (Wang et al. 2008). Taken together these findings indicate that eszopiclone + fluoxetine co-administration exerts an antidepressant neurogenic effect through mechanisms which may be independent from existing treatments.

Eszopiclone or fluoxetine alone did not significantly affect neurogenesis, although the magnitude of the fluoxetine-induced increases, ~25%, are similar to what has been observed in our previous reports (Kodama et al. 2004; Malberg et al. 2000; Nakagawa et al. 2002). The reason for this difference is not clear, as the present study used the same fluoxetine treatment regimen as in our previous reports (5 mg/kg). The higher level of variation could have been a factor.

The dorsal hippocampus has been implicated in the neurogenic effects of antidepressant treatment, and previous research has demonstrated that certain antidepressant treatments may promote cell survival, but not proliferation, in dorsal hippocampus (Banasr et al. 2006), in agreement with the effects of eszopiclone + fluoxetine co-administration reported here.

Differential roles of ventral vs. dorsal hippocampal neurogenesis is the object of an increased interest regarding antidepressant action (Sahay & Hen, 2007).

One possible mechanism for the effects of eszopiclone is the property of GABAergic excitation of immature neurons. Eszopiclone may directly act on newly proliferated cells via regulation of GABA receptor signalling, which depolarizes immature neurons, similar to the effects of GABA on developing immature neurons (Najib, 2006; Nakamichi et al. 2009), and increases neuronal survival and differentiation (Tozuka et al. 2005). Enhancement of GABAergic excitation by eszopiclone could thereby explain the effects on survival reported here. Activation of newborn cells, or of surrounding cells could also increase the expression of neurotrophic factors, such as BDNF, which has been linked to increased survival of newborn cells (Sairanen et al. 2005). Whereas the results demonstrate an antidepressant effect of eszopiclone + fluoxetine co-administration on neurogenesis, there have been clinical reports that novel hypnotic drugs, including zolpidem, zaleplon and eszopiclone, may increase the risk of developing depression and suicide (Kripke, 2007). It is also notable that zolpidem decreases neurogenesis in the adult hippocampus (Takase et al. 2009), possibly as a result of differential affinity for GABA$_A$ receptor subtypes compared to eszopiclone (Brunello et al. 2009). In addition, a recent study reports that diazepam blocks fluoxetine-induction of proliferation and does not influence survival of newborn neurons, providing further evidence that eszopiclone may have a unique binding profile (Wu & Castren, 2009).

The effects of eszopiclone + fluoxetine could also occur via regulation of other GABAergic-related mechanisms in the hippocampus, or in other brain regions associated with the behavioural actions of antidepressants, including the amygdala and prefrontal cortex. It is also possible that the effects of eszopiclone could occur via regulation of sleep, as sleep deprivation is reported to alter hippocampal neurogenesis (Grassi et al. 2006; see review Meerlo et al. 2009). Finally, the role of neurogenesis in the actions of eszopiclone + fluoxetine co-treatment needs to be tested in behaviour models that block (e.g. irradiation) or are dependent (e.g. chronic mild stress) on neurogenesis (Surget, 2008; Wojtowicz et al. 2008).

Cell proliferation and survival has been implicated in depression, antidepressant action, and the response to stress (Duman & Monteggia, 2006; Nestler et al. 2002). Results from the present study provide evidence for an antidepressant action of eszopiclone + fluoxetine co-administration on cell survival, a critical stage of neurogenesis. Additional studies will be
required to determine if the interaction between fluoxetine and eszopiclone is synergistic or additive. Because co-administration does not influence baseline cell proliferation, we hypothesize that eszopiclone treatment acts through a novel mechanism that augments existing antidepressant treatments, which primarily influence cell proliferation. The present findings provide preliminary support for a therapeutic regimen that potentiates the effects of antidepressants both on depressive symptoms and highly co-morbid disorders such as anxiety and insomnia.

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Statement of Interest
None.

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