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Glucocorticoid Receptor Antagonism Augments Fluoxetine-Induced Downregulation of the 5-HT Transporter

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The effects of combined treatment with a glucocorticoid receptor (GR) antagonist, Org 34850, and a selective serotonin reuptake inhibitor (SSRI), fluoxetine, were investigated on pre- and postsynaptic aspects of 5-HT neurotransmission. Rats were treated for 14 days with Org 34850 (15 mg per kg per day subcutaneously), fluoxetine (10 mg per kg per day intraperitoneally), or a combination of both drugs. [^{3}H]-citalopram binding (an index of 5-HT transporter (5-HTT) expression) was only slightly affected by Org 34850 alone: decreased in cortex and midbrain and increased in hippocampus. In contrast, chronic fluoxetine markedly decreased 5-HTT levels in all regions. Importantly, this decrease was significantly enhanced by combined Org 34850/fluoxetine treatment. There were no changes in the expression of 5-HTT mRNA, suggesting these effects were not due to changes in gene transcription. Expression of tryptophan hydroxylase mRNA and both 5-HT$_{1A}$ autoreceptor mRNA and protein (assessed using [^{3}H]-8-OH-DPAT binding) were unchanged by any treatment. The expression of postsynaptic 5-HT$_{1A}$ receptor protein in the forebrain was unaltered by fluoxetine, Org 34850 or the combined Org 34850/fluoxetine treatment. This downregulation of 5-HTT by fluoxetine and its enhancement by Org 34850 can explain our recent observation that GR antagonists augment the SSRI-induced increase in extracellular 5-HT. In addition, these data suggest that the augmentation of forebrain 5-HT does not result in downregulation of forebrain 5-HT$_{1A}$ receptor expression. Given the importance of 5-HT$_{1A}$ receptor-mediated transmission in the forebrain to the antidepressant response, these data indicate that co-administration of GR antagonists may be effective in augmenting the antidepressant response to SSRI treatment.
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INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) are widely used for the treatment of depression. Although effective in some patients, SSRI treatment does not always induce complete remission of depression and many patients have persistent symptoms despite apparently 'adequate' dosing (Boulenger, 2004). Interestingly, the hypothalamic–pituitary–adrenal (HPA) axis has been shown in several recent studies to have an influence on patients' response to antidepressant treatment. Thus, Young *et al* (2004) reported that the therapeutic response to SSRIs was lower in patients with HPA axis abnormalities than in those with normal HPA axis function. In a more recent and larger scale study, Brouwer *et al* (2006) showed that hyperactivity of the HPA axis predicted poor treatment response. Conversely, it has been reported that the clinical efficacy of 'serotonergic' antidepressants (including SSRIs) is improved by co-administration of an inhibitor of glucocorticoid synthesis (Jahn *et al*, 2004).

The molecular target of SSRI antidepressants is the 5-HT transporter (5-HTT), which they block competitively, preventing the reuptake of released 5-HT. In experimental animals, chronic administration of SSRIs has been shown to cause an elevation in extracellular 5-HT in the forebrain (Bosker *et al*, 1995; Dawson *et al*, 2000). It is believed that the therapeutic efficacy of SSRIs is dependent upon this elevation of forebrain 5-HT. This contention is supported by data showing that acute depletion of tryptophan (the precursor of 5-HT) results in a rapid return of symptoms in euthymic patients treated for depression with SSRIs (Delgado *et al*, 1999). An elevation of forebrain 5-HT levels would, of course, be predicted to increase the activation of postsynaptic 5-HT receptors. Although the particular receptor subtypes involved in the antidepressant response have not been definitively identified, the 5-HT$_{1A}$ receptor is thought to be important (Cowen, 2000; Celada *et al*, 2004).

As the clinical antidepressant response to SSRIs appears to be both reliant on forebrain 5-HT and influenced by corticosteroids, we proposed that glucocorticoids may modulate the ability of SSRIs to elevate forebrain 5-HT. In line with this hypothesis, we found that flattening the diurnal corticosterone rhythm in rats attenuates the ability of a chronic SSRI to elevate cortical 5-HT (Gartside et al, 2003). Importantly, recently we showed that blockade of glucocorticoid receptors (GRs) has the opposite effect, enhancing the ability of an SSRI to elevate forebrain 5-HT levels (Johnson et al, 2007). The mechanism underlying the interaction between SSRIs and glucocorticoid signaling remains unknown. However, it is of note that 5-HT neurones in the dorsal and median raphe nuclei (DRN and MRN, respectively) express GRs (Harfstrand et al, 1986; Gartside et al, 2008), which are intracellular transcription regulatory proteins. Thus, it is possible that alterations in glucocorticoid signalling lead to changes in levels of expression of particular proteins within 5-HT neurones, which ultimately influence extracellular 5-HT.

In the present study we investigated the molecular basis of the SSRI $\times$ GR antagonist interaction. To this end, we examined the effects of chronic treatment with an SSRI (fluoxetine) and/or a GR antagonist (Org 34850) on the expression of 5-HTT in the raphe and various forebrain regions considered important in the pathophysiology of depression. We also examined the expression in the raphe of mRNAs that code for the 5-HTT, the 5-HT synthesis enzyme tryptophan hydroxylase (TPH), and the 5-HT$_{1A}$ autoreceptor, as well as levels of 5-HT$_{1A}$ autoreceptor protein. Finally, to examine the consequences of the previously reported changes in 5-HT levels, we determined expression of postsynaptic 5-HT$_{1A}$ receptors in the forebrain.

MATERIALS AND METHODS

Animals

Male Hooded Lister rats (150–180 g) were purchased from Charles River, UK. They were housed in groups of three in standard caging under controlled conditions of light (lights on 0700, lights off 1900 hours) and humidity. Animals were allowed free access to standard rat chow (SDS, UK) and tap water, and allowed to acclimatize to the holding facility for at least 5 days before initiation of treatment. All experiments were carried out under the Animals (Scientific Procedures) Act 1986 in accordance with the 'Guide for the Care and Use of Laboratory Animals' promulgated by the National Institutes of Health. Animals were healthy throughout and treatment groups did not differ in weight at start or end of treatment.

Drug Treatments

Treatments were for 14 days using a 2×2 factorial design. Animals received Org 34850 (15 mg per kg, s.c.) or its vehicle (30% dimethylsulfoxide: 70% polyethylene glycol) twice daily at approximately 0700 and 1800 hours. Concurrently animals also received fluoxetine (10 mg per kg, i.p.) or its vehicle (sterile water) once daily at approximately 0700 hours. This gave four treatment groups:

vehicle/vehicle, Org/vehicle, vehicle/fluoxetine, and Org/fluoxetine.

Brain Sections

On the 15th day between 0800 and 0830 hours, animals were rapidly decapitated. The brain was removed and snap-frozen on dry ice and stored at −80°C. Coronal sections (12 µm) were cut on a cryostat, thaw-mounted onto gelatinized slides and stored at −80°C. Sections were collected from the following brain regions (anteroposterior distance from bregma according to Paxinos and Watson, 1986): midbrain (−8.0 to −7.6 mm), ventral hippocampus (−6.04 to −4.8 mm), dorsal hippocampus (−3.8 to −3.14 mm) and prefrontal cortex (PFC) (+2.2 to +3.2 mm).

In Situ Hybridization Histochemistry

In situ hybridization histochemistry with oligonucleotide probes was used to determine levels of expression of mRNAs encoding 5-HTT, 5-HT$_{1A}$ receptor and TPH in the DRN and MRN. Briefly, the sections were fixed and pretreated for in situ hybridization in a single batch using an established protocol (McQuade et al, 2004). Oligonucleotide probe sequences complimentary to bases 1345–1371 of rat 5-HTT mRNA (M79450) (5′-AGCTTCGTCTCTGGCTTC GTCATCTTC-3′) 1863–1894 of rat TPH2 mRNA (NM 173839.2) (5′-CTCACACAATTCCAGCTGCTGAGTCCTTG ACC-3′) and bases 2105–2151 of rat 5 HT$_{1A}$ receptor mRNA (AF 217200) (5′-GGTTAGCGTGGGAGGAAGGGAGACTAG CTGTCTGAGCGACATACAAG-3′) (manufactured by MWG-Biotech AG) were used. Probes were 3′ tail labelled using [^{35}S] dATP (Perkin Elmer, USA) with terminal deoxynucleotide transferase (Roche Diagnostics, West Sussex, UK). The labelled oligonucleotide probe was added to each section in hybridization buffer (50% formamide, $4 \times$ standard saline citrate (SSC), 10% dextran sulfate, $5 \times$ Denhardt's, 200 µg/ml salmon sperm DNA, 100 µg/ml polyA, 25 mM sodium phosphate, 1 mM sodium pyrophosphate, and 5% dithiothreitol (Pei et al, 1995). The sections were incubated overnight (at 38°C (5-HTT), 30°C (TPH2), and 27°C (5-HT$_{1A}$)) in sealed boxes containing 50% formamide in $4 \times$ SSC. Sections were then washed three times (20 min) in $1 \times$ SSC at 58°C and twice (60 min) at room temperature. After air-drying, the sections, together with [^{14}C] microscale calibration strips (Amersham), were exposed to Biomax Hyperfilm (Amersham) for (2–12 weeks) before automatic development using an Agfa curix compact plus daylight processor.

Binding Studies

Citalopram binding was adapted from established protocols (Romero et al, 1998; Hebert et al, 2001). The slides were preincubated in Tris buffer pH 7.4 (Tris-HCl, 50 mM, NaCl 120 mM, KCl 5 mM) at room temperature for 30 min. Slides were then incubated for 2 h at room temperature in Tris buffer containing 1.5 nM [^{3}H]-citalopram (Amersham; specific activity 84 Ci/mmol). To determine nonspecific binding, two slides from each area were incubated in 1.5 nM [^{3}H]-citalopram together with 20 µM fluoxetine. Following incubation the slides underwent two 15 min washes in

ice-cold Tris buffer, briefly washed in distilled H_2O, and left to air-dry.

The method used for determining 5-HT_{1A} binding was adapted from that used previously in this laboratory and the concentration of ligand ($[^3H]$-8-OH-DPAT) necessary for estimation of Bmax was determined from full saturation curves (C Troakes, personal communication). Slides were preincubated for 30 min at room temperature in Tris buffer pH 7.5 (Tris-HCl, 170 mM, $CaCl_2$ 4 mM, ascorbic acid 0.01%, and pargyline (10 μM; to prevent metabolism of 5-HT)). Slides were then incubated at room temperature for 90 min in fresh Tris buffer containing 2 nM $[^3H]$8-OH-DPAT (Amersham; specific activity 229 Ci/mmol). Nonspecific binding was determined for two slides per area using 5-HT (1 μM) added to the incubation medium. The slides were then washed (2×5 min) in ice-cold Tris buffer, dipped in distilled water, and air-dried.

Slides were secured inside an X-ray cassette and exposed to Biomax Hyperfilm together with a $[^3H]$ microscale calibration strip (Amersham) and left for 8–12 weeks before the film was developed using an automatic processor.

Densitometry

The density of $[^3H]$-citalopram and $[^3H]$-8-OH-DPAT binding and the relative abundance of the mRNAs were determined by densitometric quantification of quadruplicate sections using NIH Scion image software. For bilateral structures measurements were taken from both sides and averaged. Density values were calibrated to the $[^3H]$- or $[^{14}C]$ microscales, and converted to nCi/g tissue. Nonspecific binding for $[^3H]$-citalopram (defined with fluoxetine) and for $[^3H]$-8-OH-DPAT (defined with 5-HT) was negligible and was not subtracted before analysis.

Statistics

Binding data were initially analysed by three-way analysis of variance (ANOVA) with brain region treated as a repeated measure and treatment 1 (Org 34850 or vehicle) and treatment 2 (fluoxetine or vehicle) as between-subjects factors. Subsequently individual two-way ANOVA on each brain region was used, with treatments 1 and 2 as between-groups factors. Where significant interactions were found, post-hoc comparisons between individual treatment groups were made using t-test. To account for the multiple comparisons made, Bonferroni correction was applied with $p \leqslant 0.0045$ for the $[^3H]$-citalopram binding and $p \leqslant 0.0038$ for the $[^3H]$-8-OHDPAT binding being considered as statistically significant.

In situ hybridization data were subject to two-way ANOVA and, where significant interactions were found, post-hoc t-test.

RESULTS

Expression of 5-HTT in the Forebrain and Midbrain

Analysis of $[^3H]$-citalopram binding data by three-way ANOVA revealed significant main effects of 'brain region' ($F_{10,230} = 333$; $p < 0.001$) and significant brain region × 'treatment 1' ($F_{10,230} = 4.7$; $p < 0.001$) and brain region ×

'treatment 2' ($F_{10,230} = 109$; $p < 0.001$) interactions. There were also significant main effects of treatment 1 ($F_{1,23} = 14$; $p = 0.001$) and treatment 2 ($F_{1,23} = 671$; $p < 0.001$) and a significant treatment 1 × treatment 2 interaction ($F_{1,23} = 8.2$; $p = 0.009$). Further two-way ANOVAs on the individual brain regions are reported below.

Prefrontal cortex. $[^3H]$-citalopram binding was evident in a laminar distribution in the PFC, including in the cingulate, prelimbic, and infralimbic regions of the medial PFC (mPFC) (Figure 1a). In all regions of the mPFC, treatment with Org 34850 caused a small decrease ($< 15\%$), whereas fluoxetine treatment caused a marked reduction (approximately 60%) in $[^3H]$-citalopram binding. When fluoxetine and Org 34850 treatments were combined, $[^3H]$-citalopram binding was even further reduced and in some animals was close to the limit of detection (Figure 2). Two-way ANOVA showed a significant effect of fluoxetine and a significant effect of Org 34850, but there was no significant interaction between the treatments (Table 1; Figure 3).

Dorsal hippocampus. In the dorsal hippocampus $[^3H]$-citalopram binding was dense in Ammon's horn with the highest density in stratum oriens of CA2 at the apex of the horn. Much lower binding was seen in the dentate gyrus, which could not be anatomically discriminated (Figure 1b). In Ammon's horn treatment with Org 34850 alone caused a very small increase in $[^3H]$-citalopram binding. In all subregions, treatment with chronic fluoxetine alone markedly reduced $[^3H]$-citalopram binding. However, in animals treated with the combination of fluoxetine and Org 34850, $[^3H]$-citalopram binding was reduced much more than in animals treated with fluoxetine alone (Figure 2). Two-way ANOVAs in each subregion revealed significant main effect of fluoxetine in all subregions, and a significant Org 34850 × fluoxetine interaction in CA2 and CA3 (Table 1; Figure 3). Post-hoc t-test revealed that $[^3H]$-citalopram binding in the Org 34850/fluoxetine group was significantly lower than in the fluoxetine-alone group.

Ventral hippocampus. In the ventral hippocampus $[^3H]$-citalopram binding could clearly be seen in Ammon's horn with much lower expression in dentate gyrus (not measured) (Figure 1c). In the group treated with Org 34850 alone, $[^3H]$-citalopram binding was slightly increased relative to the vehicle/vehicle group. Treatment with fluoxetine alone decreased $[^3H]$-citalopram binding in Ammon's horn. However, in the animals treated with the combination of Org 34850 and fluoxetine, $[^3H]$-citalopram binding was reduced below the level seen after fluoxetine alone (Figure 2). Two-way ANOVA revealed a significant main effect of fluoxetine and a significant Org 34850 × fluoxetine interaction in all subregions (Table 1; Figure 3). Post-hoc t-test revealed that in all subregions $[^3H]$-citalopram binding in the Org 34850/fluoxetine group was significantly lower than in the fluoxetine-alone group.

Midbrain. In the midbrain $[^3H]$-citalopram binding was densely expressed in the raphe nuclei (Figure 1d). In both the DRN and MRN, chronic Org 34850 treatment slightly reduced $[^3H]$-citalopram binding. Chronic fluoxetine
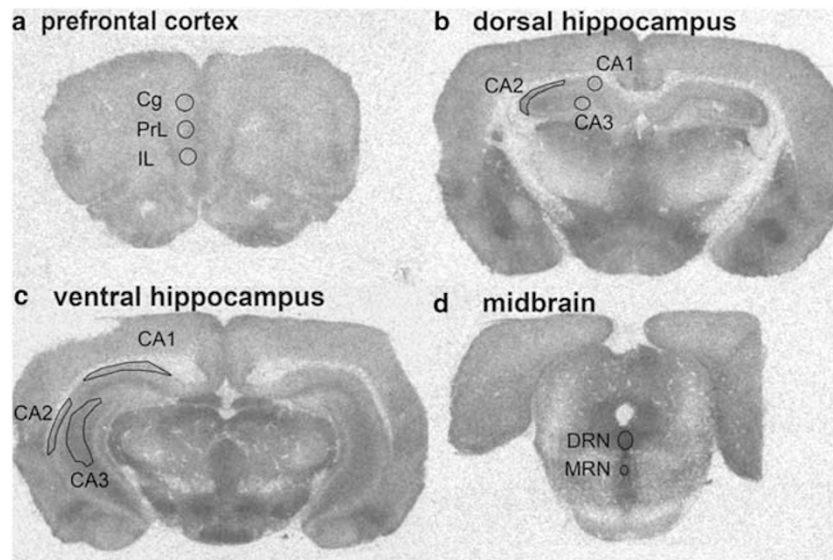
Figure 1 Autoradiograms showing [3H] citalopram binding in prefrontal cortex (a), dorsal hippocampus (b), ventral hippocampus (c) and midbrain raphe nuclei (d) of vehicle/vehicle-treated animals. Regions analysed by densitometry are labeled: Cg, cingulate cortex; PrL, prelimbic cortex; IL, infralimbic cortex; CA1-CA3, Cornus Ammon 1–3; DG, dentate gyrus; DRN, dorsal raphe nucleus; MRN, median raphe nucleus.

treatment caused a marked reduction in [3H]-citalopram binding in both nuclei (Figure 2). In the group treated with fluoxetine and Org 34850 combined, [3H]-citalopram binding was even more reduced. Two-way ANOVA in the DRN and the MRN showed significant main effects of Org 34850 and fluoxetine but no significant interaction between treatments (Table 1; Figure 3).

Expression of mRNAs Coding for 5-HTT, TPH and 5-HT$_{1A}$ Autoreceptor in the DRN

The mRNAs coding for 5-HTT, TPH and the 5-HT$_{1A}$ autoreceptor were highly and selectively expressed in the raphe nuclei. Within the midbrain sections examined signal was restricted to the DRN and adjacent ventral MRN. There were no significant effects of Org 34850 or fluoxetine or the fluoxetine/Org 34850 combination on the levels of expression of mRNAs coding for 5-HTT, TPH, or the 5-HT$_{1A}$ autoreceptor in the DRN (Table 1; Figure 4). Thus, two-way ANOVA showed no significant main effects and no significant interactions.

Effects of Org 34850 and Fluoxetine on 5-HT$_{1A}$ Receptor Protein

Analysis of [3H]-8-OHDPAT binding data by three-way ANOVA revealed a significant main effect of brain region ($F_{12,288} = 972$; $p < 0.001$), and a significant brain region × treatment 2 interaction ($F_{12,288} = 2.2$; $p = 0.011$). However, there was no significant effect of treatment 1 ($F_{1,24} = 0$; n.s.) or treatment 2 ($F_{1,24} = 0$; n.s.) and no significant treatment 1 × treatment 2 interaction ($F_{1,24} = 1.4$; n.s.). Further two-way ANOVAs on the individual brain regions are reported below.

Prefrontal cortex. In sections containing the PFC, [3H]-8-OH-DPAT binding was present in the cortex but not the corpus callosum or anterior commissure (Figure 5a). Within the cortex, the distribution was laminar being lowest in superficial layers and highest in layers V and VI. In the mPFC binding was particularly dense in layer VI adjacent to the corpus callosum. There were no significant effects of Org 34850 or fluoxetine treatment on [3H]-8-OH-DPAT binding in any parts of the PFC and no interaction between treatments (Table 1; Figure 6).

Dorsal hippocampus. In the dorsal hippocampus [3H]-8-OH-DPAT binding was dense in stratum oriens and stratum radiatum dendritic layers of Ammon's horn, but was much lower in the pyramidal cell layer (Figure 5b). Binding in CA2 was markedly lower than in the adjacent CA1 and binding in CA3 was higher than in CA1. In the dentate gyrus, [3H]-8-OH-DPAT binding was high in the molecular layer (which contains the dendrites of granule cells) but was low in the granule cell layer. Neither Org 34850 nor fluoxetine treatment had any effect on [3H]-8-OH-DPAT binding in any area and there was no interaction between Org 34850 and fluoxetine treatments (Table 1; Figure 6).

Ventral hippocampus. The distribution of [3H]-8-OH-DPAT binding in the ventral (caudal) hippocampus was similar to that in the dorsal (rostral) hippocampus being lowest in CA2 and highest in CA3 and the molecular layer of the dentate gyrus. A further dense area of binding was seen in the most ventral part of CA1 (Figure 5c). In all subregions of the ventral hippocampus [3H]-8-OH-DPAT binding was unaffected by Org 34850 or fluoxetine treatment, as well as by the combined Org 34850/fluoxetine treatment (Table 1; Figure 6).

Midbrain raphe. In midbrain sections containing the anterior raphe nuclei, [3H]-8-OH-DPAT binding was present in the DRN and MRN (Figure 5d). There were no
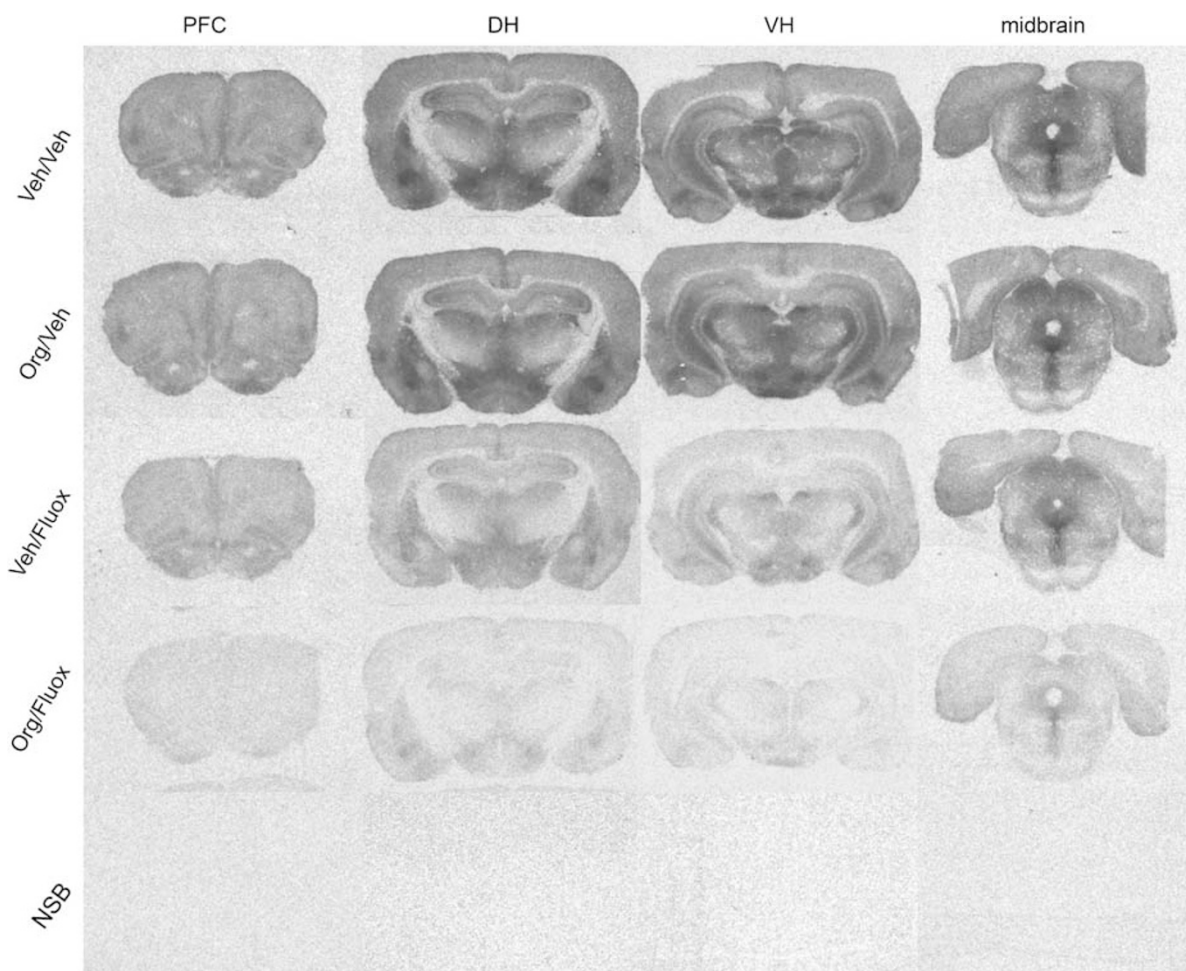
Figure 2 Autoradiograms showing [^{3}H]-citalopram binding in prefrontal cortex (PFC), dorsal hippocampus (DH), ventral hippocampus (VH) and midbrain in animals treated with vehicle/vehicle (Veh/Veh), Org 34850/vehicle (Org/Veh), vehicle/fluoxetine (Veh/Fluox) and Org 34850/fluoxetine (Org/Fluox). The final row shows nonspecific binding (NSB) in each region. Autoradiograms shown are from individual animals displaying [^{3}H]-citalopram binding densities typical of each treatment group.

significant effects of Org 34850 or fluoxetine treatment on [^{3}H]-8-OH-DPAT binding in the DRN or MRN and no interaction between the treatments (Table 1; Figure 6).

DISCUSSION

Here we investigated the effects of chronic fluoxetine and Org 34850 treatment, alone and in combination, on the expression of mRNAs and proteins associated with pre- and postsynaptic aspects of 5-HT neurotransmission. Chronic fluoxetine treatment caused a striking decrease in [^{3}H]-citalopram binding (a measure of 5-HTT expression) in cortex and hippocampus, as well as in the raphe nuclei. Importantly, concurrent Org 34850 and fluoxetine treatment markedly enhanced this decrease in 5-HTT levels, whereas Org 34850 treatment alone caused only small (and in some cases opposing) effects on 5-HTT. There were no changes in the expression of mRNAs coding 5-HTT, TPH, or the 5-HT$_{1A}$ receptor, suggesting these effects were not mediated through changes in gene transcription. Furthermore, there were no significant effects of any of the treatments on either 5-HT$_{1A}$ autoreceptor or postsynaptic

5-HT$_{1A}$ receptor levels (measured by [^{3}H]-8-OH-DPAT binding). Org 34850 is a steroid molecule (for structure see Bachmann et al, 2003), which has highest affinity for the GR (pEC$_{50}$ = 8.0) with 3-fold selectivity over progesterone receptors and more than 300-fold selectivity over MR (Organon Laboratories in-house data). Our results indicate that the ability of GR antagonists (including Org 34850) to augment the effect of an SSRI on forebrain 5-HT (Johnson et al, 2007) results from a widespread enhancement of the fluoxetine-induced downregulation of 5-HTT. This augmentation of extracellular 5-HT appears not to cause any marked change in forebrain 5-HT$_{1A}$ receptor expression. Given the proposed importance of 5-HT$_{1A}$ receptor-mediated transmission to the antidepressant response, these data indicate that co-administration of GR antagonists may effectively augment the antidepressant response to SSRI treatment.

Downregulation of the 5-HTT

Chronic fluoxetine treatment induced a marked reduction in 5-HTT expression. Several studies have reported that chronic administration of other SSRIs decreases the Bmax

Table 1 Statistical Analysis

[³H]-Citalopram binding	d.f.	Org 34850		Fluoxetine		Interaction	
		F	*p*	F	*p*	F	*p*
Prefrontal cortex							
Cingulate	1,28	14.3	**0.001**	464.8	**<0.0001**	0.5	0.509
Prelimbic	1,28	12.4	**0.002**	234.3	**<0.0001**	0.0	0.840
Infralimbic	1,28	12.2	**0.002**	362.9	**<0.0001**	0.5	0.471
Dorsal hippocampus							
CA1	1,26	2.7	0.111	216.7	**<0.0001**	4.8	0.038
CA2	1,26	7.1	0.013	378.2	**<0.0001**	8.6	**0.007**
CA3	1,26	6.4	0.018	362.6	**<0.0001**	10.9	**0.003**
Ventral hippocampus							
CA1	1,26	0.7	0.398	119.8	**<0.0001**	10.1	**0.004**
CA2	1,27	2.9	0.103	329.4	**<0.0001**	11.8	**0.002**
CA3	1,27	0.9	0.340	208.3	**<0.0001**	10.5	**0.003**
Midbrain							
DRN	1,26	19.8	**<0.0001**	416.0	**<0.0001**	0.7	0.420
MRN	1,28	21.0	**0.0001**	346.1	**<0.0001**	0.8	0.391
5-HTT mRNA							
DRN	1,27	1.2	0.282	0.5	0.469	1.7	0.202
TPH₂ mRNA							
DRN	1,25	0.4	0.539	0.0	0.979	1.4	0.243
5-HT₁ₐ mRNA							
DRN	1,26	0.2	0.643	0.5	0.469	0.0	0.899
[³H]-8-OH-DPAT binding							
Prefrontal cortex							
Cingulate	1,27	0.7	0.404	2.3	0.142	0.3	0.570
Prelimbic	1,27	4.1	0.052	0.0	0.975	3.4	0.078
Infralimbic	1,28	1.3	0.258	0.0	0.864	2.6	0.117
Dorsal hippocampus							
CA1	1,27	1.6	0.221	2.5	0.122	0.5	0.493
CA2	1,27	0.0	0.891	1.4	0.256	3.8	0.063
CA3	1,27	2.6	0.119	6.4	0.017	0.9	0.345
DG	1,27	0.5	0.471	8.7	0.007	3.5	0.071
Ventral hippocampus							
CA1	1,27	0.3	0.625	2.9	0.099	2.5	0.127
CA2	1,26	0.2	0.784	0.3	0.592	4.1	0.053
CA3	1,26	1.2	0.287	0.0	0.957	3.6	0.068
DG	1,26	0.5	0.483	0.2	0.706	1.5	0.227
Midbrain							
DRN	1,28	0.7	0.417	2.4	0.135	0.2	0.655
MRN	1,28	0.5	0.829	2.6	0.120	0.3	0.580

Abbreviations: DRN, dorsal raphe nuclei; MRN, median raphe nuclei; 5-HTT, 5-HT transporter; TPH, tryptophan hydroxylase; DG, dentate gyrus.
Results of two-way ANOVA with fluoxetine and Org 34850 as factors. 'Org 34850' refers to the main effect of Org 34850 administration, 'Fluoxetine' refers to the main effect of fluoxetine administration and 'interaction' refers to the Org 34850 × fluoxetine interaction. Degrees of freedom (d.f.), F ratio and the raw *p*-value for each analysis are given. Figures in bold type are statistically significant ($p < 0.0045$) following Bonferroni correction for multiple comparisons.
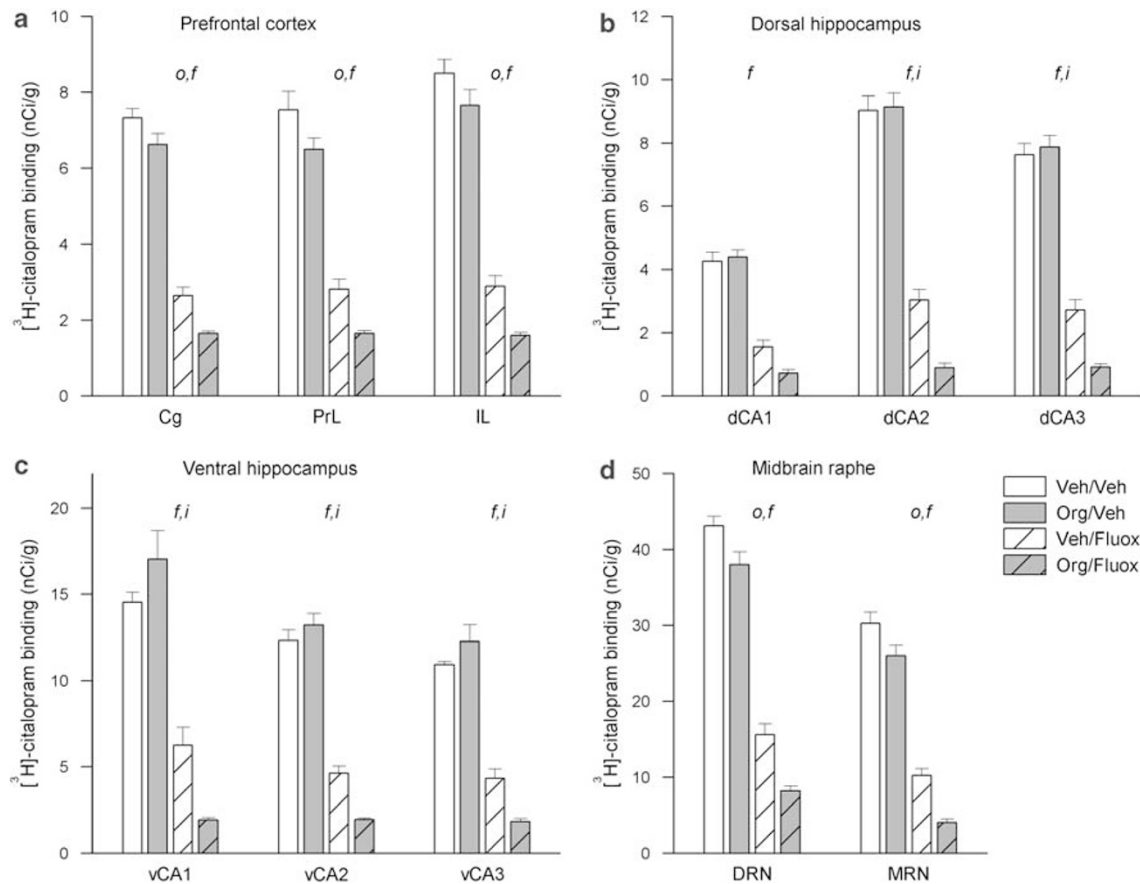
Figure 3 Density of [^{3}H]-citalopram binding in subregions of the medial prefrontal cortex (mPFC) (a), dorsal hippocampus (b), ventral hippocampus (c) and midbrain raphe (d) in animals treated with vehicle/vehicle, Org 34850/vehicle, vehicle/fluoxetine and Org 34850/fluoxetine. Cg, cingulate cortex; PrL, prelimbic cortex; IL, infralimbic cortex; dCA1-3, dorsal Cornus Ammon 1–3; vCA1-3, ventral Cornus Ammon 1–3. Data are mean ± SEM. N = 7 or 8 per group; o denotes significant effect of Org 34850, f significant main effect of fluoxetine, and i significant interaction. See text and Table 1 for full details of statistical analysis.

of 5-HTT binding (Pineyro et al, 1994; Benmansour et al, 1999, 2002; Gould et al, 2006). Moreover, in complete accord with the present findings, Durand et al (1999) demonstrated a marked decrease in [^{3}H]-citalopram binding following chronic treatment with fluoxetine. Although other reports suggest that chronic fluoxetine fails to decrease 5-HTT levels (Dewar et al, 1993; Gobbi et al, 1997; Hebert et al, 2001), the low doses of fluoxetine and/or lengthy washout periods used may account for these discrepancies. In the present study we measured 5-HTT binding in brains taken around 24 h after the last of 14 daily injections of fluoxetine (at the same point as we had made our measures of 5-HT levels (Johnson et al, 2007)), and our binding protocol included a room temperature wash step to remove any residual drug.

The principal finding of this study is Org 34850 treatment enhanced the downregulation of the 5-HTT induced by fluoxetine. In the PFC and raphe nuclei, Org 34850 had an additive effect with fluoxetine reducing 5-HTT more than either treatment alone. However, in the hippocampus Org 34850 alone had no effect or tended to increase 5-HTT but, when combined with fluoxetine, displayed a marked synergy reducing 5-HTT to very low levels. Downregulation of 5-HTT may have a very important role in the delayed emergence of the neurochemical and antidepressant effects

of SSRIs. Thus, Benmansour and colleagues showed that the SSRI-induced decrease in 5-HTT has a much more marked effect on extracellular 5-HT than does the presence of the reuptake inhibitor itself (Benmansour et al, 2002). Perhaps because during acute blockade, the dynamic competition between the reuptake inhibitor and the increasing levels of extracellular 5-HT limits the consequences whereas, following downregulation of the 5-HTT (ie a loss of 5-HTT protein from the membrane) no such competition can take place. Org 34850 in combination with fluoxetine leads to a greater downregulation of 5-HTT, and so might be expected to lead to a greater enhancement of extracellular 5-HT levels, than fluoxetine alone. Thus, the biochemical data presented here are consistent with in vivo microdialysis findings in which we observed that concurrent treatment with Org 34850 enhanced the effect of fluoxetine on extracellular 5-HT in the PFC (Johnson et al, 2007). However, it is worth noting that although the present data showed Org 34850 alone caused a small reduction in 5-HTT in the PFC, this treatment failed to affect extracellular 5-HT levels. One explanation for this is that there is spare transporter capacity and so the modest reduction in 5-HTT induced by Org 34850 alone is of insufficient magnitude to elevate 5-HT levels.

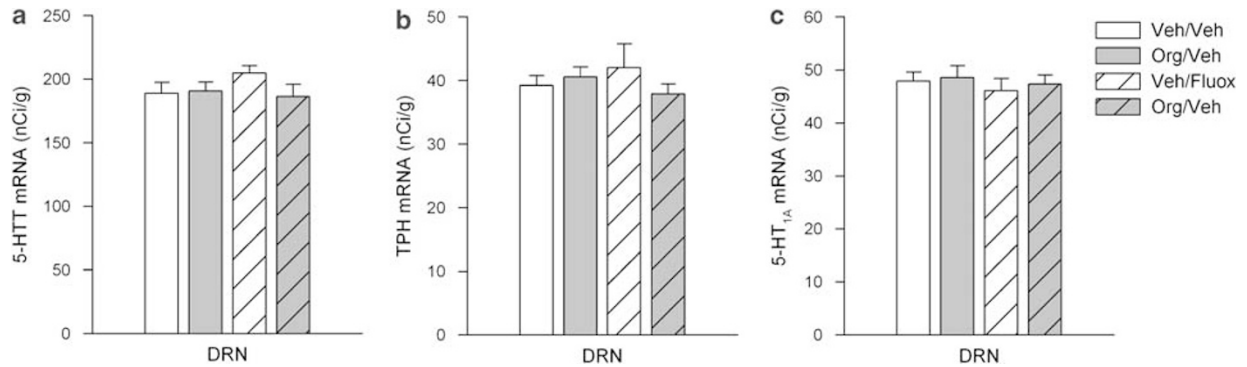In addition to providing a mechanistic explanation for our previous findings (Johnson et al, 2007), the present data

Figure 4 Density of expression of mRNAs for 5-HT transporter (5-HTT) (a), tryptophan hydroxylase (TPH) (b) and 5-HT$_{1A}$ receptor (c) in the dorsal raphe nuclei (DRN) in animals treated with vehicle/vehicle, Org 34850/vehicle, vehicle/fluoxetine and Org 34850/fluoxetine. Data are mean ± SEM. $N = 7/8$ per group. See text and Table 1 for full details of statistical analysis.
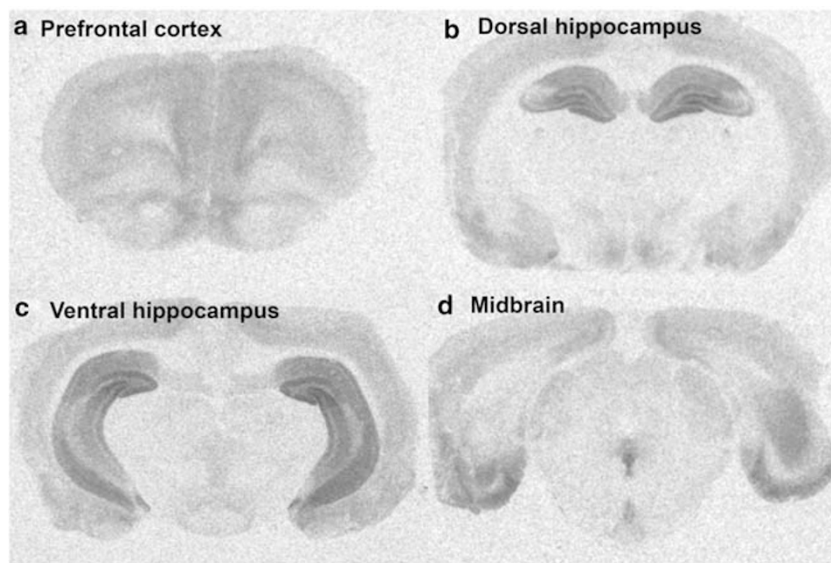

Figure 5 Autoradiograms showing distribution and relative densities of [^{3}H]-8-OH-DPAT binding in prefrontal cortex (a), dorsal hippocampus (b), ventral hippocampus (c) and midbrain raphe nuclei (d). All autoradiograms are from animals treated with vehicle/vehicle and are typical of the group.

suggest that the augmentation of the effect of an SSRI on 5-HT would not be confined to the PFC (where our microdialysis was performed) but would occur widely through the brain. Indeed, in both dorsal and ventral hippocampus we found evidence for a true synergy between fluoxetine and Org 34850, which would predict a synergistic interaction in respect of extracellular 5-HT levels.

The fact that GRs are transcription regulatory proteins raises the possibility that the effect of the GR antagonist might be mediated by changes in mRNA expression. GRs have been shown to be expressed in 5-HT neurones in both the DRN and MRN (Harfstrand *et al*, 1986), thereby having the potential to regulate protein synthesis within 5-HT neurones. However, none of the treatments affected expression of 5-HTT mRNA in the raphe nuclei. Although the effect of GR antagonists on 5-HTT mRNA has not been previously studied, the lack of effect of fluoxetine is consistent with previous data showing that chronic treatment with the same dose of fluoxetine did not alter 5-HTT mRNA measured at 21 days (Neumaier *et al*, 1996; Le Poul *et al*, 2000).

TPH2 mRNA expression was also unaltered by any of the treatments, suggesting that the alterations in extracellular 5-HT following fluoxetine and its enhancement by GR antagonists (Johnson *et al*, 2007) does not arise from a transcriptional changes in the TPH2 gene.

5-HT$_{1A}$ Autoreceptors

None of the treatments altered the mRNA coding the 5-HT$_{1A}$ autoreceptor in the DRN or the 5-HT$_{1A}$ protein in either DRN or MRN. We have previously found functional desensitization of the somatodendritic 5-HT$_{1A}$ autoreceptor following chronic fluoxetine (Gartside *et al*, 2003; Johnson *et al*, 2007). The present data indicate that this desensitization is not the result of a decrease in 5-HT$_{1A}$ receptor gene transcription or protein levels. This concurs with previous studies reporting fluoxetine-induced desensitization in 5-HT$_{1A}$ autoreceptor function without concomitant changes in receptor binding (Le Poul *et al*, 1995; Hanoun *et al*, 2004).
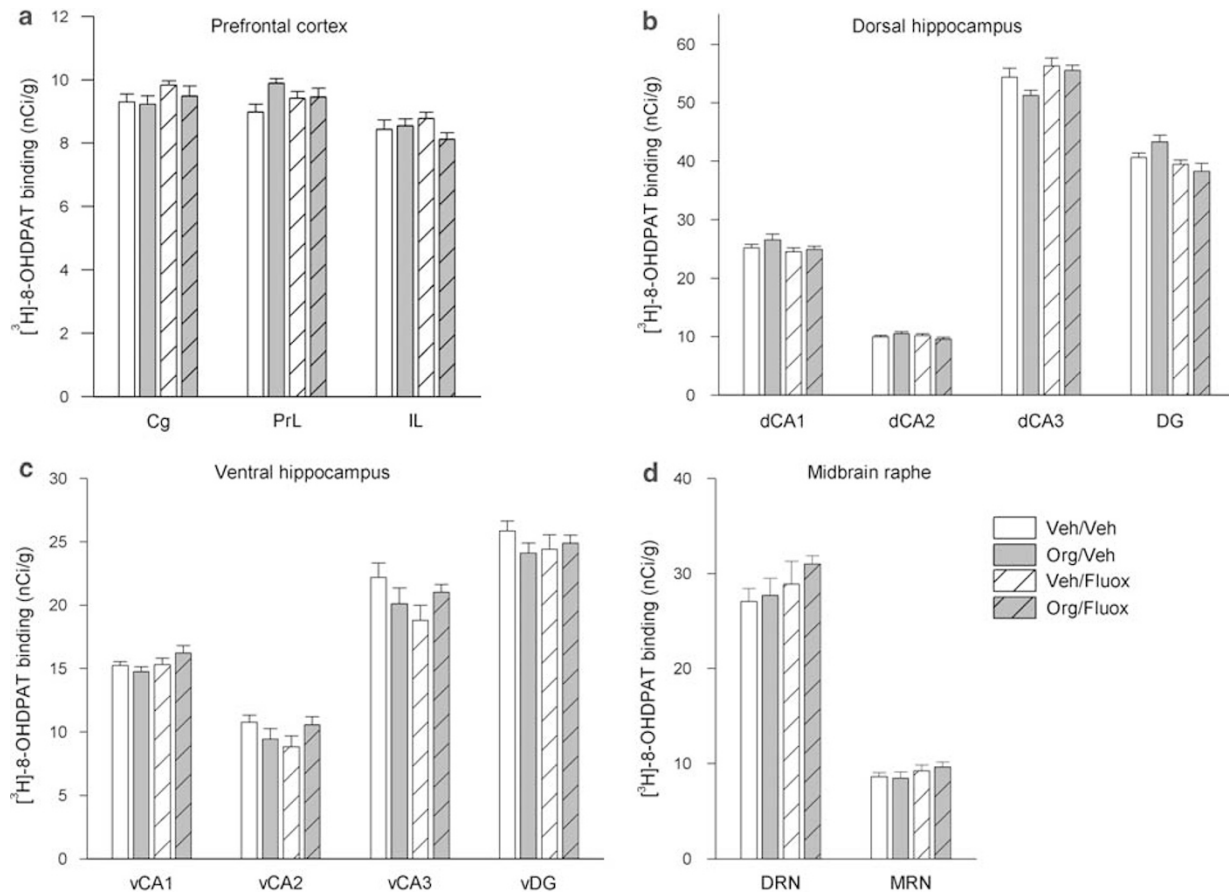
Figure 6 Density of [³H]-8-OH-DPAT binding in subregions of the prefrontal cortex (PFC) (a), the dorsal hippocampus (b), the ventral hippocampus (c), and the dorsal and median raphe nuclei (DRN/MRN) (d) in animals treated with vehicle/vehicle, Org 34850/vehicle, vehicle/fluoxetine and Org 34850/fluoxetine. Cg, cingulate cortex; PrL, prelimbic cortex; IL, infralimbic cortex; dCA1-3, dorsal Cornus Ammon 1–3; dDG, dorsal dentate gyrus; vCA1-3, ventral Cornus Ammon 1–3; vDG, ventral dentate gyrus. Data are mean ± SEM. N = 7 or 8 per group; o denotes significant effect of Org 34850, f significant main effect of fluoxetine, and i significant interaction. See text and Table 1 for full details of statistical analysis.

Postsynaptic 5-HT$_{1A}$ Receptors

Postsynaptic 5-HT$_{1A}$ receptors, in both hippocampus and PFC, have been hypothesized to be important in the antidepressant response (Cowen, 2000; Celada et al, 2004). For this reason we examined the effect of chronic SSRI and Org 34850 treatment on the expression of this receptor. Neither Org 34850 nor fluoxetine significantly altered 8-OH-DPAT binding across all forebrain regions measured. The lack of effect of fluoxetine is in line with previous reports that chronic administration of SSRIs fails to alter 5-HT$_{1A}$ receptor binding in forebrain (Li et al, 1993; Le Poul et al, 2000; Hensler, 2002). Consistent with this, in depressed patients, postsynaptic 5-HT$_{1A}$ receptor binding measured by positron emission tomography was recently reported to be unchanged by chronic SSRI treatment (Moses-Kolko et al, 2007). The effects of chronic GR antagonist treatment on 5-HT$_{1A}$ binding have not been examined previously, however, 5-HT$_{1A}$ binding is reportedly unchanged in GR deficient mice compared to wild types (Meijer et al, 1997). Crucially, in the present study we found that the combination of fluoxetine and Org 34850 also failed to alter 5-HT$_{1A}$ receptor binding. This suggests that the marked increase in extracellular 5-HT that arises from combined SSRI and GR

antagonist treatment (Johnson et al, 2007) does not lead to changes in the number of postsynaptic 5-HT$_{1A}$ receptors.

Although 5-HT$_{1A}$ receptor number was not decreased the treatments, it cannot be assumed that 5-HT$_{1A}$ receptor function is unaltered. That said, much evidence suggests that chronic SSRI treatment does not affect postsynaptic 5-HT$_{1A}$ receptor sensitivity (Dong et al, 1999; Le Poul et al, 2000; Li et al, 1997; Hensler, 2002). Indeed, as chronic administration of direct 5-HT$_{1A}$ receptor agonists has been shown not to alter 5-HT$_{1A}$ receptor sensitivity in CA3 (Dong et al, 1997; Haddjeri et al, 1999), one can conclude that these receptors are unresponsive to agonist-induced down-regulation or desensitization. Thus, although the combined treatment with fluoxetine and Org 34850 causes a marked increase in extracellular 5-HT (Johnson et al, 2007), we would predict that this would not alter 5-HT$_{1A}$ receptor sensitivity.

Conclusion

In our previous study we found that co-treatment with GR antagonists enhanced the SSRI-induced increase in extracellular 5-HT in the PFC (Johnson et al, 2007). Here we show that the mechanism underlying this interaction is an

augmentation by the GR antagonist of the fluoxetine-induced downregulation of 5-HTT. Moreover, this enhancement of transporter downregulation is widespread, suggesting that GR antagonists have the potential to enhance SSRI effects on extracellular 5-HT in many brain areas. We have also shown that combined GR antagonist/SSRI treatment does not result in a decrease in 5-HT$_{1A}$ receptor protein and have presented evidence that argues against there being a downstream functional desensitization of these receptors. Thus, we predict that the overall effect of co-administration of a GR antagonist and an SSRI will be a marked enhancement of forebrain 5-HT$_{1A}$ receptor-mediated neurotransmission. It has been hypothesized that an antidepressant response arises from increased postsynaptic 5-HT$_{1A}$ receptor-mediated transmission in the forebrain (Cowen, 2000; Celada *et al*, 2004). Hence, our data indicate that co-administration of GR antagonists may effectively augment the antidepressant response to SSRI treatment.

DISCLOSURE/CONFLICT OF INTEREST

This study was supported by Organon Laboratories under an investigator-initiated grant award to Newcastle University. Dr Emma Grant and Dr Mark Craighead are employees of Organon Laboratories. Professor Colin Ingram and Dr Sarah Gartside are co-applicants on patent applications relating to therapeutic uses of GR antagonists.

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