Clozapine and olanzapine, but not haloperidol, suppress serotonin efflux in the medial prefrontal cortex elicited by phencyclidine and ketamine

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Abstract

N-methyl-D-aspartate (NMDA) receptor antagonists such as phencyclidine (PCP) and ketamine can evoke psychotic symptoms in normal individuals and schizophrenic patients. Here, we have examined the effects of PCP (5 mg/kg) and ketamine (25 mg/kg) on the efflux of serotonin (5-HT) in the medial prefrontal cortex (mPFC) and their possible blockade by the antipsychotics, clozapine, olanzapine and haloperidol, as well as ritanserin (5-HT2A/2C receptor antagonist) and prazosin (α1-adrenoceptor antagonist). The systemic administration, but not the local perfusion, of the two NMDA receptor antagonists markedly increased the efflux of 5-HT in the mPFC. The atypical antipsychotics clozapine (1 mg/kg) and olanzapine (1 mg/kg), and prazosin (0.3 mg/kg), but not the classical antipsychotic haloperidol (1 mg/kg), reversed the PCP- and ketamine-induced increase in 5-HT efflux. Ritanserin (5 mg/kg) was able to reverse only the effect of PCP. These findings indicate that an increased serotonergic transmission in the mPFC is a functional consequence of NMDA receptor hypofunction and this effect is blocked by atypical antipsychotic drugs.

Received 24 March 2005; Reviewed 18 April 2005; Revised 15 June 2005; Accepted 15 June 2005; First published online 15 August 2005

Key words: Clozapine, haloperidol, ketamine, phencyclidine, serotonin.

Introduction

The hypothesis of the N-methyl-D-aspartate (NMDA) receptor hypofunction in schizophrenia stems from the observation that NMDA receptor antagonists such as phencyclidine (PCP) and ketamine can evoke psychotic symptoms in normal individuals and aggravate them in schizophrenic patients (for review, see Krystal et al., 2003). For these reasons, NMDA receptor antagonists have been used in experimental animals to model neurological and behavioural changes that occur in schizophrenia. Thus, systemic administration of PCP or dizocilpine (MK-801) increases the firing of neurons of the medial prefrontal cortex (mPFC) and causes hyperlocomotion and stereotypies (Jackson et al., 2004; Jodo et al., 2003; Suzuki et al., 2002). However, microiontophoretically applied PCP does not alter either mPFC cell firing or hyperlocomotion (Suzuki et al., 2002), which suggests that the NMDA receptor antagonists turn on mPFC neurons through the stimulation of excitatory inputs from brain areas outside the mPFC. NMDA receptor antagonists have also been shown to increase extracellular glutamate (Abekawa et al., 2003; Lorrain et al., 2003; Moghadam et al., 1997), dopamine (Adams and Moghadam, 1998; Mathe` et al., 1999; Schmidt and Fadayel, 1996), and serotonin (5-HT) (Adams and Moghadam, 2001; Martin et al., 1998a; Millan et al., 1999) in the mPFC. The source of this glutamate is presently unknown, but it has been proposed that NMDA receptor antagonists may block a tonic inhibitory influence of γ-aminobutyric acid (GABA) neurons over an excitatory projection to the mPFC (Jodo et al., 2005; Krystal et al., 2003; Lorrain et al., 2003; Moghadam et al., 1997). The increases in dopamine and 5-HT release are probably due to increments in dopaminergic (Pawlowski et al., 1990; Schmidt and Fadayel, 1996) and serotonergic (Lejeune et al., 1994)
cell firing in the ventral tegmental area and the dorsal raphe nucleus respectively. This latter activation could result from an enhanced glutamatergic output from the mPFC neurons, including those projecting to the dorsal raphe nucleus. Indeed, the electrical or pharmacological stimulation of the mPFC increases serotonergic transmission in the dorsal raphe nucleus (Celada et al., 2001; Martín-Ruiz et al., 2001). Nevertheless, a local effect of NMDA receptor antagonists in the dorsal raphe nucleus cannot be ruled out (Callado et al., 2000).

Although a hyperactive serotonergic transmission in the mPFC has been implicated in schizophrenia (Meltzer, 1989) and in the hyperlocomotion induced by a reduced NMDA receptor function (Miyamoto et al., 2001), the precise role of cortical 5-HT on these effects remains to be determined. However, a relationship between 5-HT and schizophrenia stems from two main observations. First, 5-HT2A/2C receptor agonists elicit hallucinogenic states in humans resembling those present in positive symptomatology (Gouzoulis-Mayfrank et al., 1998), and markedly increase the firing rate of pyramidal neurons in the mPFC (Puig et al., 2003). Likewise, the selective activation of 5-HT2A receptors in rodent mPFC increases local 5-HT release (Amargós-Bosch et al., 2004; Bortolozzi et al., 2003; Martín-Ruiz et al., 2001; Puig et al., 2003), which could point to an activation of mPFC serotonergic transmission in the illness. Second, atypical antipsychotics are effective 5-HT2A receptor antagonists. In previous studies we have shown that antipsychotic drugs reverse the increase of the local extracellular 5-HT induced by the pharmacological stimulation of the mPFC with 5-HT2A and α1-adrenergic receptor agonists (Amargós-Bosch et al., 2003; Bortolozzi et al., 2003). In the present work, we have examined the effects of clozapine, olanzapine and haloperidol on the efflux of 5-HT in the mPFC elicited by PCP and ketamine. In an attempt to understand the contribution of 5-HT2A/2C and α1-adrenergic receptors in the action of these antipsychotic drugs, the effects of ritanserin and prazosin were also tested.

### Materials and methods

#### Animals

Male Wistar rats (Iffa-Credo, Lyon, France) weighing 250–280 g were used. They were maintained on a 12-h light/dark cycle (lights on at 07:00 hours) and housed three per cage before surgery and individually after surgery. Food and water were always freely available. All experimental procedures were carried out in strict accordance with European Communities Council Directive on ‘Protection of Animals Used in Experimental and Other Scientific Purposes’ of 24 November 1986 (86/609/EEC) and were approved by the Institutional Animal Care and Use Committees.

#### Drugs

Phencyclidine hydrochloride, ritanserin and prazosin hydrochloride were purchased from Sigma-Aldrich (Tres Cantos, Spain). Ketamine hydrochloride (Ketolar®, 50 mg/ml) and haloperidol were purchased as injectable solutions from Pfizer and Laboratorios Esteve (Barcelona, Spain) respectively. Clozapine was obtained from Tocris (Bristol, UK). Olanzapine and citalopram hydrobromide were generously donated by Eli Lilly & Co (Indianapolis, IN, USA) and H. Lundbeck A/S (Copenhagen-Valby, Denmark) respectively. PCP and Ketolar® were dissolved in distilled water or artificial cerebrospinal fluid according to the route of administration. Clozapine, olanzapine, prazosin and ritanserin were dissolved in a few drops of glacial acetic acid and further diluted with distilled water for systemic administration, or artificial cerebrospinal fluid (see below for composition) for local application through dialysis probes. When needed, the pH of the final concentrations was adjusted to 6.5–7.0 with NaHCO3. Because PCP and ketamine were administered intraperitoneally (i.p.) and subcutaneously (s.c.) respectively, two different control groups were run in parallel. One group consisted of a first s.c. injection followed 20 min later by an i.p. injection of saline and served as control for the experiment with PCP. The other group consisted of two s.c. injections of saline and served as control for the ketamine experiment. When antipsychotics, prazosin and ritanserin were administered alone, a single s.c. injection of saline served as control group.

#### Microdialysis procedures

Concentric dialysis probes with a 4-mm long membrane were implanted under sodium pentobarbital anaesthesia (60 mg/kg i.p.) in the mPFC (AP +3.2 mm, L −0.8 mm, DV −6.0 mm; from Bregma) according to Paxinos and Watson (1986). Microdialysis experiments were conducted 20–24 h after surgery in freely moving rats by continuously perfusing probes with artificial cerebrospinal fluid containing 125 mM NaCl, 2.5 mM KCl, 1.26 mM CaCl2, 1.18 mM MgCl2 and 1 μM citalopram, at a rate of 1.5 μl/min. Dialysate samples of 30 μl were collected every 20 min. After a 100-min stabilization period, four dialysate samples were collected to obtain basal 5-HT values before...
pharmacological treatment. At the completion of dialysis experiments, rats were given an overdose of sodium pentobarbital and a Fast Green solution was perfused through the dialysis probes to stain the surrounding tissue.

**Biochemical determinations**

The concentration of 5-HT in dialysate samples was determined by HPLC using a 3-μm octadecylsilica (ODS) column (7.5 cm × 0.46 cm; Beckman, San Ramon, CA, USA) and detected amperometrically with a Hewlett-Packard 1049 detector (Palo Alto, CA, USA) set at an oxidation potential of 0.6 V. The detection limit for 5-HT was estimated to be \( \sim 1 \) fmol/sample.

**Experimental design**

Basically, each experimental group started with the collection of four dialysate samples before drug administration (basal values). Then, a systemic injection (i.p. or s.c.) of saline or a drug (clozapine, olanzapine, haloperidol, prazosin or ritanserin), followed by the administration of a NMDA receptor antagonist (PCP or ketamine) or saline. To examine the local effects of administration of a NMDA receptor antagonist (PCP (i.p. or s.c.) or saline or a drug (clozapine, olanzapine, haloperidol, prazosin or ritanserin)), followed by the administration of a NMDA receptor antagonist (PCP or ketamine) or saline. To examine the local effects of NMDA receptor antagonists on extracellular 5-HT, successive increasing concentrations of PCP or ketamine were perfused for 80 min following the collection of four basal dialysate samples. The rats of the corresponding control group were perfused only with artificial cerebrospinal fluid.

**Statistics**

Data (mean ± S.E.M.) are expressed as fmol/fraction (uncorrected for recovery) and shown in figures as percentages of basal values, averaged from four fractions collected before treatment. The changes in dialysate 5-HT were analysed by two-way repeated-measures analysis of variance (ANOVA) with time and drug as factors. Area under the curve (AUC) was calculated as percentage from the same period of control sample.

**Results**

The basal (pre-drug) concentration of 5-HT was not significantly different among all experimental groups and amounted to 37.6 ± 1.7 fmol/sample (\( n = 126 \)). The perfusion of PCP (10, 30, 100, 300 μM) or ketamine (100, 300, 1000 μM) in the mPFC did not change the local concentration of 5-HT (Figure 1). There was no difference between the three control groups used with regard to dialysate 5-HT. As described thoroughly in the literature, the systemic administration of PCP (5 mg/kg i.p.) and ketamine (25 mg/kg s.c.) to rats evoked a marked hyperlocomotion and stereotypies (behavioural observation). The concentration of 5-HT was also elevated significantly following the systemic administration of PCP \( ( F_{1,12} = 17.28, p < 0.002) \) and ketamine \( ( F_{1,12} = 16.26, p < 0.002) \). The effect of PCP was \( \sim 20\% \) higher than that of ketamine, but this difference did not reach statistical significance. The PCP-induced increase of 5-HT was suppressed by clozapine 1.0 mg/kg s.c. \( ( F_{1,11} = 10.79, p < 0.01) \), olanzapine 1.0 mg/kg s.c. \( ( F_{1,14} = 5.24, p < 0.05) \), ritanserin 5.0 mg/kg i.p. \( ( F_{1,11} = 5.12, p < 0.05) \) and prazosin 0.3 mg/kg s.c. \( ( F_{1,9} = 14.08, p < 0.005) \), but not by haloperidol 0.1 mg/kg and 1.0 mg/kg s.c. (Figure 2). In a similar way, the ketamine-induced increase of 5-HT was abolished by clozapine 1.0 mg/kg s.c. \( ( F_{1,12} = 12.92, p < 0.005) \), olanzapine 1.0 mg/kg s.c. \( ( F_{1,14} = 5.61, p < 0.05) \) and prazosin 0.3 mg/kg s.c. \( ( F_{1,12} = 30.66, p < 0.0002) \), but not by haloperidol 1.0 mg/kg s.c. and ritanserin 5.0 mg/kg i.p. (Figure 3). When injected alone (Figure 4), clozapine 1.0 mg/kg s.c. \( ( F_{1,9} = 8.38, p < 0.02) \), haloperidol 1.0 mg/kg s.c. \( ( F_{1,12} = 6.39, p < 0.05) \) and prazosin 0.3 mg/kg s.c. \( ( F_{1,9} = 15.67, p < 0.005) \), reduced mPFC 5-HT to the same extent (\( \sim 70\% \) of baseline values). In contrast, ritanserin 5.0 mg/kg i.p. resulted in a 20%
increase \( F_{1,1} = 10.05, p < 0.01 \) and olanzapine 1.0 mg/kg s.c. produced no change in dialysate 5-HT. It was of note that none of these drugs evoked any gross behavioural change when administered alone (behavioural observation).

**Discussion**

The first main finding of this study is that the systemic administration of the NMDA receptor antagonists, PCP and ketamine, increases the efflux of 5-HT in the mPFC. To the best of our knowledge, this was previously shown for PCP (Adams and Moghaddam, 2001; Martin et al., 1998a; Millan et al., 1999), but not for ketamine. Likewise, previous work had shown increases in 5-hydroxyindoleacetic acid (5-HIAA) following MK-801 administration (Kashiwa et al., 1995; Lindefors et al., 1997; Lösch er et al., 1993), which could be indicative of an enhanced 5-HT metabolism and/or release. Altogether these findings indicate that an increased serotonergic transmission in the mPFC is a general response to NMDA receptor hypofunction. There does not seem to be differences between competitive and non-competitive NMDA receptor antagonists since both classes of compounds produce a similar activation of cortical serotonergic pathways (Lösch er et al., 1993) and 5-HT release (Ceglia et al., 2004). Furthermore, this effect does not result from a direct action of these compounds on the mPFC because the local application of PCP or ketamine did not produce any change in the concentration of 5-HT. In line with our results, the intra-mPFC administration of NMDA receptor antagonists does not affect the local neuronal activity (Aghajanian and Marek, 2000; Jodo et al., 2003; Suzuki et al., 2002), which suggests that the NMDA receptors responsible for such actions are located outside the mPFC. One possible site of action of NMDA receptor antagonists is the ventral hippocampus because the local application of PCP or MK-801 in this region increases the firing of pyramidal neurons in the mPFC (Jodo et al., 2005). According to the hypothesis of the disinhibition of glutamatergic input to the mPFC (see Introduction), PCP and ketamine would increase glutamate release onto

olanzapine 1.0 mg/kg s.c. (OLZ, \( n = 8 \)) and prazosin 0.3 mg/kg, s.c. (PRZ, \( n = 4 \)), but not by haloperidol 0.1 mg/kg s.c. (HAL 0.1, \( n = 4 \)), haloperidol 1.0 mg/kg s.c. (HAL 1, \( n = 5 \)) or ritanserin 5.0 mg/kg i.p. (RIT, \( n = 5 \)). \( * p < 0.05 \) compared to SAL + PCP group and \( \# p < 0.05 \) compared to SAL + SAL group.

**Figure 2.** Effects of phencyclidine (PCP) 5 mg/kg i.p. on the 5-HT efflux in the mPFC alone (\( n = 8 \)) or in combination with antipsychotic drugs (a) or receptor antagonists (b). Data (mean \( \pm \) S.E.M.) in (a) and (b) are expressed as percentage of baseline. The group SAL + PCP in (a) has been replotted in (b). Data in (c) represent the area under the curve (AUC) of the different treatments. PCP increased dialysate 5-HT with respect to the control group (\( n = 6 \)) that received two injections of saline (SAL). The effect of PCP was blocked by pretreatment with clozapine 1.0 mg/kg s.c. (CLZ, \( n = 5 \)),
The stimulation of such non-NMDA receptors would, in turn, produce an enhanced glutamatergic output from the mPFC neurons, including those projecting to the dorsal raphe nucleus, thereby enhancing serotonergic cell firing and cortical 5-HT efflux. Therefore, the observed effects on 5-HT efflux would be secondary to a primary release of glutamate in the mPFC. In line with these results, the release of 5-HT in the mPFC evoked by the 5-HT$_{2A/2C}$ receptor agonist 1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane (DOI) or the $\alpha_1$-adrenoceptor agonist cirazoline was reversed by the $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate receptor blockade and by the activation of mGluR2/3 receptors (Amargós-Bosch et al., 2003; Bortolozzi et al., 2003; Martín-Ruiz et al., 2001). However, although this functional interplay between the mPFC and the dorsal raphe nucleus is well documented (Amargós-Bosch et al., 2003; Celada et al., 2001; Hajós et al., 1998; Lucas et al., 2005; Martín-Ruiz et al., 2001), a direct influence of NMDA receptor antagonists on serotonergic neurons at the level of the dorsal raphe nucleus cannot be discarded. Indeed, the local application of MK-801 into the dorsal raphe nucleus increases the local release of 5-HT both in vitro (Callado et al., 2000) and in vivo (Tao and Auerbach, 2000). A third alternative could be that the NMDA receptor antagonist-induced excess of cortical glutamate would act on AMPA receptors presumably located on serotonergic terminals in the mPFC, thus promoting 5-HT release. Several reports have described the presence of such AMPA receptors in the presynaptic compartment with a role in the regulation of transmitter release (Schenk and Matteoli, 2004). Moreover, the existence of presynaptic AMPA receptors in serotonergic nerve endings has been proposed in other regions of the brain (Maione et al., 1997) although it remains to be determined in the mPFC.

The second important finding of the present study is that the increased efflux of 5-HT produced by the systemic administration of PCP and ketamine was reversed by the atypical antipsychotics clozapine and olanzapine, but not by the classical antipsychotic haloperidol. The blocking effect of olanzapine was injections of saline (SAL). The effect of ketamine was blocked by pretreatment with clozapine 1.0 mg/kg s.c. (CLZ, $n=5$), olanzapine 1.0 mg/kg s.c. (OLZ, $n=7$) and prazosin 0.3 mg/kg s.c. (PRZ, $n=5$), but not by haloperidol 1.0 mg/kg s.c. (HAL 1, $n=4$) or ritanserin 5.0 mg/kg i.p. (RIT, $n=4$).

* $p<0.05$ compared to SAL + KET group and # $p<0.05$ compared to SAL + SAL group.

Figure 3. Effects of ketamine (KET) 25 mg/kg s.c. on the 5-HT efflux in the mPFC alone ($n=9$) or in combination with antipsychotic drugs (a) or receptor antagonists (b). Data (mean ± S.E.M.) in (a) and (b) are expressed as percentage of baseline. The group SAL + KET in (a) has been replotted in (b). Data in (c) represent the area under the curve (AUC) of the different treatments. Ketamine increased dialysate 5-HT with respect to the control group ($n=5$) that received two
delayed in comparison with that of clozapine, which may be related to differences in kinetics of the drugs. In general, atypical antipsychotics have been shown to be more effective than classical antipsychotics in reversing behavioural deficits (for review, see Jentsch and Roth, 1999) and the blockade of NMDA responses on pyramidal neurons of the mPFC (Wang and Liang, 1998) elicited by PCP. It remains to be determined, however, if the effects of clozapine and olanzapine are dependent on cortical glutamate changes. Previous research appeared to point in that direction inasmuch as clozapine tended to block glutamate efflux induced by PCP (Adams and Moghaddam, 2001). However, the high variability of the responses precluded any definitive conclusion. On the other hand, the effects of PCP and ketamine were antagonized by the selective α1-adrenoceptor antagonist prazosin. Both clozapine and haloperidol possess a similar affinity for the α1-adrenoceptor (Arnt and Skarsfeldt, 1998; Millan et al., 1998) and together with olanzapine are able to inhibit serotonergic cell firing in the dorsal raphe nucleus through α1-adrenoceptor antagonism (Millan et al., 1998; Sprouse et al., 1999). Furthermore, clozapine and haloperidol occupy a similar amount (~50%) of cortical α1-adrenoceptors at the doses used in the present work (Chaki et al., 1999). Altogether these findings would suggest that, at the doses tested, the partial occupancy of α1-adrenoceptors is not responsible for the different effects of these drugs on the efflux of 5-HT and that these effects would not result from an inhibition of serotonergic cell firing but rather from a direct cortical action. In contrast, the dose of prazosin fully occupies brain α1-adrenoceptors (Patel et al., 2001). Thus, its effects may result not only from a post-synaptic action in the mPFC since its local application in the mPFC markedly reduced 5-HT release (Amargós-Bosch et al., 2003), but also from its strong inhibition of serotonergic cell firing at the raphe level (Baraban and Aghajanian, 1980). Nevertheless, it should be kept in mind that the sole blockade of α1-adrenoceptors does not posses antipsychotic action.

In addition to α1-adrenoceptors, clozapine and olanzapine exhibit affinities much greater than that of haloperidol for 5-HT2C receptors (Arnt and Skarsfeldt, 1998; Bymaster et al., 1996). However, antagonism at 5-HT2C receptors increases serotonergic transmission. Thus, a selective 5-HT2A receptor antagonist (SB 242084) is able to potentiate cortical 5-HT release elicited by 5-HT reuptake blockade (Cremers et al., 2004).

This is probably the mechanism by which ritanserin slightly but significantly increased mPFC 5-HT in the present work.

Finally, clozapine and olanzapine share a high affinity for the 5-HT1A receptor (Arnt and Skarsfeldt, 1998; Bymaster et al., 1996), which suggests that this receptor could theoretically play a role in the action of these drugs in reducing the increased efflux of 5-HT evoked by PCP and ketamine. In fact, the 5-HT1A receptor antagonist ritanserin was able to abolish the effects of PCP on the 5-HT efflux, which can be predominantly attributed to its 5-HT1A antagonistic properties (see above). In accord with our results, the selective 5-HT1A receptor antagonist, M100907, offsets the PCP-induced hyperlocomotion, although PCP does not directly interact with 5-HT1A receptors (Millan et al., 1999). In addition, the pharmacological stimulation of the mPFC with S-AMPA is also
reversed by 5-HT\textsubscript{2A} receptor antagonists (Amargós-Bosch et al., 2003), which suggests that the 5-HT\textsubscript{2A} antagonism is able to counteract an excessive stimulation of AMPA receptors in the mPFC. However, ritanserin did not antagonize the ketamine-induced increase in 5-HT efflux, despite it being somewhat lower than that produced by PCP. One possibility of reconciling these apparently discordant findings is that 5-HT\textsubscript{1A/AC} receptor blockade may be effective only in conditions of a high serotonergic tone. In accordance with this, M100907 can block MK-801-induced hyperlocomotion, but not after 5-HT depletion (Martin et al., 1998b). The precise location of the 5-HT\textsubscript{1A} receptors responsible for these effects remains to be determined. It is of significance that, although M100907 is able to block the effects of 5-HT\textsubscript{2A} receptor agonists at the level of the dorsal raphe nucleus (Boothman et al., 2003), the evidence that 5-HT cells do not express 5-HT\textsubscript{1A} receptors (Cornea-Hebert et al., 1999; Pompeiano et al., 1994) further suggests that post-synaptic (possibly cortical) 5-HT\textsubscript{1A} receptors are involved.

Regardless of the precise mechanism, it seems that the ability to prevent the NMDA receptor antagonist-induced enhancement of 5-HT efflux in the mPFC may be a characteristic of atypical antipsychotic drugs. At the dose of 1 mg/kg, it is possible that occupancy of both \(\alpha_1\)-adrenergic and 5-HT\textsubscript{2A} receptors by clozapine and olanzapine may contribute to the blockade of the NMDA receptor antagonism-induced increase in cortical 5-HT efflux. Given that an overactive serotonergic transmission has been inferred to occur in schizophrenia, a reduction of 5-HT in the mPFC may be suggestive of a better antipsychotic profile for atypical antipsychotic drugs. At 5-HT\textsubscript{2A} doses of 1 mg/kg, it is possible that occupancy of both \(\alpha_1\)-adrenergic and 5-HT\textsubscript{2A} receptors by clozapine and olanzapine may contribute to the blockade of the NMDA receptor antagonism-induced increase in cortical 5-HT efflux. Given that an overactive serotonergic transmission has been inferred to occur in schizophrenia, a reduction of 5-HT in the mPFC may be suggestive of a better antipsychotic profile for negative-cognitive symptoms. It must be taken into consideration, however, that the effects observed in the present work are limited to the pharmacological profiles of clozapine and olanzapine. While all classical antipsychotics are characterized by a predominant \(\text{D}_2\) receptor antagonism, within the atypical class each drug possesses a distinct pharmacological profile. It is, therefore, difficult to assign the blockade of NMDA receptor antagonist-induced release of cortical 5-HT to a particular receptor interaction, although the 5-HT\textsubscript{2A} receptor seems to have an important contribution.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Education and Science (SAF2004-05525 and the CIEN Network (IDIBAPS-ISCIII RTIC C03/06). M.A.-B. and X.L.-G. are recipients of predoctoral fellowships from the Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) and Ministry of Education and Science respectively. The excellent technical assistance of Leticia Campa is greatly appreciated. Thanks are also due to Eli Lilly & Co and H. Lundbeck A/S for their generous supply of drugs.

Statement of Interest

None.

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