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The 5-HT_6 Receptor Antagonist SB-271046 Selectively Enhances Excitatory Neurotransmission in the Rat Frontal Cortex and Hippocampus

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Preclinical evidence has suggested a possible role for the 5-HT_6 receptor in the treatment of cognitive dysfunction. However, currently there is little neurochemical evidence suggesting the mechanism(s) which may be involved. Using the selective 5-HT_6 antagonist SB-271046 and in vivo microdialysis, we have evaluated the effects of this compound on the modulation of basal neurotransmitter release within multiple brain regions of the freely moving rat. SB-271046 produced no change in basal levels of dopamine (DA), norepinephrine (NE) or 5-HT in the striatum, frontal cortex, dorsal hippocampus or nucleus accumbens. Similarly, this compound had no effect on excitatory neurotransmission in the striatum or nucleus accumbens. Conversely, SB-271046 produced 3- and 2-fold increases in extracellular glutamate levels in both frontal cortex and dorsal hippocampus, respectively. These effects were completely attenuated by infusion of tetrodotoxin but unaffected by the muscarinic antagonist, atropine. Here we demonstrate for the first time the selective enhancement of excitatory neurotransmission by SB-271046 in those brain regions implicated in cognitive and memory function, and provide mechanistic evidence in support of a possible therapeutic role for 5-HT_6 receptor antagonists in the treatment of cognitive and memory dysfunction.

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KEY WORDS: *5-HT_6 receptor; SB-271046; Microdialysis; Glutamate; Striatum; Frontal cortex; Dorsal hippocampus; Nucleus accumbens; Cognition*

The 5-hydroxytryptamine (5-HT, serotonin) receptor superfamily currently consists of 14 members divided into seven classes (5-$HT_{1\text{-}7}$) according to structural and functional homologies (for review see Barnes and Sharp 1999). One of the most recently identified of these is the 5-HT_6 receptor. Initially cloned from rat striatum (Monsma et al. 1993; Ruat et al. 1993), the 5-HT_6 receptor, like 5-HT_4 and 5-HT_7, is a G protein-linked receptor, which stimulates adenylate cyclase via G_s – coupling. *In situ* hybridization and Northern blot studies have revealed that 5-HT_6 receptor mRNA appears to be almost exclusively expressed within the brain (Monsma et al. 1993; Ruat et al. 1993; Ward et al. 1995). Regional analysis of expression reveals that the highest levels of mRNA are found within the olfactory tubercle, striatum, nucleus accumbens, cerebral cortex and subfields of the hippocampus (Monsma et al. 1993; Ruat et al. 1993; Gerard et al. 1996). Similarly, Gerard et al. (1997) showed a comparable distribution of protein using polyclonal antibodies raised to a presumed unique portion of the C terminus of the receptor. These studies also revealed that 5-HT_6 receptor expression appears to be present within 5-HT projection fields and not in 5-HT neurons

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of the raphe, indicating a probable postsynaptic role for this receptor (Ward et al. 1995; Gerard et al. 1997).

The exact functional role of the 5-HT_6 receptor has yet to be ascertained; however, its distribution together with its high affinity (nM) for many of the therapeutically effective antipsychotic and antidepressant drugs suggests possible therapeutic roles in both schizophrenia and depression (Monsma et al. 1993; Roth et al. 1994). Early experiments showed that administration of antisense oligonucleotides into the brain induced a behavioral syndrome, which could be blocked by the muscarinic antagonist, atropine (Bourson et al. 1995). A further study showed that antisense oligonucleotide treatment failed to alter the gross behavior of animals during a conditioned fear stress paradigm (a model of anxiety), but an attenuation of anxiety-induced prefrontal cortex 5-HT release was observed (Yoshioka et al. 1998). 5-HT_6 knockout mice have also been reported and these animals do not appear to have any marked phenotypic abnormalities, but do display some increase in anxiety in the elevated zero-maze (Tecott et al. 1998). More recently, Bentley et al. (1999) and Bourson et al. (1998) have demonstrated that the selective antagonist Ro 04-6790 can induce stretching and inhibit 6-OHDPAT lesion-induced rotational behaviors. Both these phenomena can be blocked by the application of muscarinic antagonists. Routledge et al. (1999) observed a potentiation of physostigmine-induced chewing behavior by SB-271046 and the same compound has been shown to be effective in enhancing cognitive function in models of learning and memory (Rogers et al. 1999). Taken together these data indicate that the 5-HT_6 receptors may be involved in the modulation of cholinergic function, suggesting a possible therapeutic utility in the treatment of memory and cognitive dysfunction.

Although learning and memory has been suggested as a therapeutic target, very little neurochemical data has been reported in support of this hypothesis. A recent abstract communication demonstrated increases in extracellular levels of acetylcholine within both the cortex and hippocampus following administration of the selective antagonist Ro 65-7199, at doses shown to be effective in behavioral models of memory deficit (Sleight et al. 1999). Furthermore, we have recently reported preliminary observations demonstrating increases in extracellular levels of glutamate within the frontal cortex by SB-271046 (Dawson et al. 2000). Therefore, to more fully elucidate the neurochemical mechanism responsible for the observed cognitive enhancement, we have examined the role of the 5-HT_6 receptor in the modulation of multiple neurotransmitters in those brain regions shown to have the highest receptor expression levels (Monsma et al. 1993; Ruat et al. 1993; Ward et al. 1995; Gerard et al. 1997). Using the potent, selective and bioavailable 5-HT_6 antagonist 5-Chloro-N-(4-methoxy-3-piperazin-1-yl-phenyl)-3-methyl-2-benzothiophenesulfon- amide (SB-271046; Bromidge et al. 1999) and *in vivo* microdialysis, we have evaluated the effects of this compound on the modulation of basal 5-HT, dopamine (DA), norepinephrine (NE) and glutamate (Glu) release within multiple brain regions in the freely moving rat.

METHODS

Materials

All chemicals used were analytical grade and were purchased from Aldrich & Sigma chemicals (Milwaukee, WI, USA). Tetrodotoxin (TTX) was purchased from Alamone labs (Jerusalem, Israel). Atropine was purchased from Research Biochemical International (Natick, MA, USA). (5-Chloro-N-(4-methoxy-3-piperazin-1-yl-phenyl)-3-methyl-2-benzothiophenesulfonamide (SB-271046) was synthesized by Chemical Sciences, Wyeth Ayerst Research (Princeton, NJ, USA).

Animals

Male Sprague-Dawley rats (280–350 g, Charles River Laboratories, Wilmington, MA) were used in all experiments (n = 8–14 per study group). Animals were group housed in cages with food and water available *ad libitum.* Following surgery, the animals were singly housed in Plexiglass cages (45 × 45 × 30 cm) with food and water available *ad libitum.* All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health.

Surgical procedure

Following induction of anesthesia, with gaseous administration of halothane (3%) (Fluothane, Zeneca, Cheshire, UK), the animals were secured in a stereotaxic frame with ear and incisor bars. Anesthesia was maintained by continuous administration of halothane (1–2%). A microdialysis probe guide cannula (CMA/Microdialysis, Stockholm, Sweden) was implanted into either the striatum (RC +0.2, L–3, V–3), frontal cortex (RC+3.5, L-3.2, V-1.5), dorsal hippocampus (RC-4.3, L-2.6, V-2.1) or nucleus accumbens (RC+2.2, L-1.4, V-6.0). Co-ordinates were taken according to Paxinos and Watson (1986) with reference points taken from bregma and vertical from the skull. A subcutaneous cannula (s.c.) was also implanted at this time between the animal's shoulders. Both cannula were secured to the skull using dental acrylic (Plastics One, Roanoke, VA, USA). The wound was sutured and the animals left to recover for 18–24 h in their home cages with free access to food and water.

Microdialysis

A pre-equilibrated microdialysis probe was inserted into the guide cannula, in either the striatum (O.D. 0.5 mm, membrane length 4 mm; CMA/Microdialysis, Sweden), frontal cortex, dorsal hippocampus or nucleus accumbens (O.D. 0.5 mm, membrane length 2 mm; CMA/Microdialysis, Sweden) of the unrestrained rat, post surgery. The probe was perfused with artificial cerebrospinal fluid (aCSF; NaCl 125 mM, KCl 3.0 mM, $MgSO_4$ 0.75 mM, $CaCl_2$ 1.2 mM and 0.1 M phosphate buffer pH 7.4) at a flow rate of 1.0 μl/min. A 3 h stabilization period was allowed following probe implantation, after which time microdialysis sampling was carried out by a modification of the method of Dawson and Nguyen (1998). Four baseline samples were taken prior to drug injection to achieve a steady baseline. These four samples were averaged and all subsequent values were expressed as a percentage of this preinjection value. SB-271046 (10 mg/kg), atropine (3 mg/kg) or vehicle was administered via the s.c. cannula. TTX (10μM) was infused, via reverse microdialysis, through the probe for a period of 40 min (t = 180–220). A 20-min sampling regime was used throughout the experimental period. At the end of the experiment probe placement was verified histologically and data from animals with incorrect probe placement were discarded.

Analysis of Microdialysates

Microdialysates were split and taken for amino acid analysis and monoamine/catacholamine determinations as follows:

1) NE, DA and 5-HT were separated by reverse phase high performance liquid chromatography (HPLC) (C18 ODS2 column, 100 x 3.0 mm, Metachem, Torrance, CA, USA) and detected using an ANTEC electrochemical detector (ANTEC, Netherlands) set at a potential of 0.7V vs an Ag/AgCl reference electrode. Mobile phase was delivered by a Jasco PU980 HPLC pump (Jasco Ltd, Essex, UK) at 0.5 ml/min and contained 0.15 M NaH_2PO_4 buffer at pH 4.3, 0.25 mM EDTA, 1.5 mM 1-octane sodium sulphonic acid and 5% isopropanol.

2) Measurement of glutamate was performed using a Crystal 310 capillary electrophoresis system (Thermo BioAnalysis, NM, USA) with a Zeta laser induced fluorescence detector (ZETA Technology, Toulouse, France) coupled with a Helium-Cadmium laser (Em-442 nm; Omnichrome, CA, USA). All samples were pre-derivatized with naphthalene 2,3-dicarboxaldehyde (NDA) by a modification of the method of Hernandez et al. (1993). Dialysate or standard samples (3 μl) were mixed with 50 mM boric acid buffer pH 9.5 containing 20 mM sodium cyanide (5 μl) and 30 mM NDA in methanol (1 μl). Samples were allowed to react for 3 min at room temperature prior to injection. Separations were performed according to Dawson et al. (1997) in fused silica capillaries (75 μm id, 375 μm od, 47 cm; Polymicro technologies, NM, USA) with an applied voltage of 0.6 kV/cm. Samples (5 nl) were applied to the capillary via a high pressure injection system. Separations used 30 mM boric acid pH 9.5 (pH adjusted using 1 M NaOH). The capillary was rinsed with 0.1 M NaOH (1.5 min) and running buffer (1.5 min) between analyses.

All data were acquired using the Atlas software package (Thermo Labsystems, Gulph Mills, PA) for the PC.

Data Analysis

The fmol/μM perfusate values of transmitters/amino acids for the first four baseline samples were averaged and this value denoted as 100%. Subsequent sample values were expressed as a percentage of this preinjection control value. Results were analyzed by analysis of variance with repeated measures followed by pairwise comparisons using Bonferroni adjustment for multiple comparisons using the Statview software application (Abacus Concepts Inc., Berkeley, CA 1996) for the PC.

RESULTS

Effects of SB-271046 on Extracellular Levels of 5-HT, NE and DA in the Striatum, Frontal Cortex, Dorsal Hippocampus or Nucleus Accumbens of the Freely Moving Rat

Subcutaneous injection of 10 mg/kg SB-271046 produced no significant change in extracellular concentrations of 5-HT, NE or DA within the striatum, frontal cortex, dorsal hippocampus or nucleus accumbens of the freely moving rat (Table 1) for up to 240 min post-administration. A small increase in extracellular DA was observed in the frontal cortex (Table 1); however, this effect failed to reach statistical significance.

Effects of SB-271046 on Extracellular Levels of Glutamate in the Striatum, Frontal Cortex, Dorsal Hippocampus or Nucleus Accumbens of the Freely Moving Rat

Subcutaneous injection of 10 mg/kg SB-271046 produced no significant change in extracellular concentrations of glutamate in the striatum (Table 1). In contrast, the same dose of SB-271046 induced a significant ($p < .05$) increase in extracellular glutamate concentrations in both the frontal cortex and the dorsal hippocampus reaching a maximum values of 375.4 ± 82.3 % (t = 280 min) and 217.8 ± 34.8 % (t = 280 min) of preinjection values, respectively (Table 1). A smaller increase in extracellular glutamate was also observed in the nucleus

Table 1. Effects of SB-271046 (10 mg/kg s.c.) on Extracellular Levels of Neurotransmitters in Various Brain Regions. Data Expressed as Maximum Observed % of Preinjection Levels Mean ± S.E.M. (n = 8–14 Per Group). Also Shown are the Basal Levels of NE, DA and 5-HT (Expressed as Mean ± S.E.M. fmol/10 μl Microdialysate) and Glu (Expressed as Mean ± S.E.M. μM/Microdialysate) from Each Brain Region. * Denotes Statistical Difference ($p < 0.05$) between SB-271046 and Vehicle Treated Animals

	NE	DA	5-HT	Glu
Frontal Cortex				
Basal	11.1 ± 0.2	14.1 ± 0.25	9.7 ± 0.2	1.08 ± 0.02
Vehicle	97.0 ± 14.3	102.1 ± 6.7	98.2 ± 11.0	99.2 ± 17.5
SB-271046	96.9 ± 28.7	133.2 ± 22.9	103.5 ± 14.8	375.4 ± 82.3*
Striatum				
Basal	37.0 ± 1.3	38.2 ± 0.8	8.8 ± 0.2	1.09 ± 0.03
Vehicle	111.0 ± 24.5	96.2 ± 18.8	102.4 ± 14.9	115.5 ± 11.5
SB-271046	129.0 ± 24.1	125.4 ± 19.2	78.5 ± 16.9	104.3 ± 25.8
Hippocampus				
Basal	3.44 ± 0.23	4.6 ± 0.27	13.8 ± 1.37	1.87 ± 0.12
Vehicle	103.6 ± 25.1	108.7 ± 14.5	104.4 ± 27.2	98.8 ± 20.3
SB-271046	142.2 ± 19.2	113.8 ± 13.2	100.2 ± 27.9	217.8 ± 34.8*
N. Accumbens				
Basal	15.5 ± 1.30	33.83 ± 2.0	5.86 ± 0.37	0.52 ± 0.02
Vehicle	106.2 ± 31.5	103.7 ± 9.9	92.4 ± 26.2	124.7 ± 5.60
SB-271046	122.1 ± 25.5	97.8 ± 2.7	134.4 ± 25.9	175.9 ± 24.8

accumbens; however, this effect failed to reach statistical significance (Table 1).

Effects of Tetrodotoxin (10 μM) and Atropine (3 mg/kg s.c.) on SB-271046-induced Increases in Extracellular Glutamate in the Frontal Cortex and Dorsal Hippocampus

Infusion of the voltage-dependent Na^+ channel blocker, tetrodotoxin (TTX; 10 μM), produced no change in basal levels of excitatory amino acid in either brain region examined (Figures 1 and 2). Alternatively, following the initial SB-271046-induced increases, infusion of TTX (t = 180–220 min) resulted in a significant attenuation in extracellular glutamate levels reducing maximal levels to 185.0 ± 12.4 % (t = 260 min; Fig. 1) and 125.5 ± 26.5 % (t = 280 min; Fig. 2) for the frontal cortex and hippocampus, respectively.

Administration of the muscarinic antagonists, atropine (3 mg/kg s.c.) had no significant effect on basal levels of glutamate in either the frontal cortex (Figure 3) or dorsal hippocampus (Figure 4). Similarly, atropine produced no significant change in SB-271046-induced increases in glutamate in either brain region (Figures 3 and 4).

DISCUSSION

The 5-HT_6 receptor is the most recently identified of the 5-HT receptor subtypes (Monsma et al. 1993; Ruat et al. 1993) and although the exact role of this receptor has not been fully elucidated, evidence is accumulating to suggest possible functions. Much of the early data, involving administration of antisense oligonucleotides and more recently with the development of selective antagonists, have suggested a role for the 5-HT_6 receptor in the modulation of cholinergic function. These data, taken together with behavioral observations demonstrating utility in models of cognitive impairment (Rogers et al. 1999; Sleight et al. 1999), have lead to the hypothesis that 5-HT_6 antagonists may have therapeutic utility in the treatment of cognitive and memory

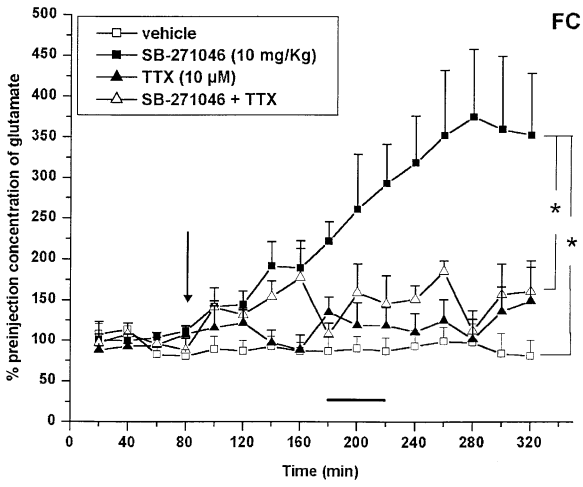

Figure 1. Effects of SB-271046 (10 mg/kg s.c) on extracellular levels of glutamate in the frontal cortex of the freely moving rat. Data expressed as mean ± S.E.M, (n = 8–14 per study group). Arrow denotes subcutaneous drug or vehicle injection points; solid bar denotes TTX (10 μM) or vehicle infusion. * denotes statistical significance ($p < .05$) between groups.

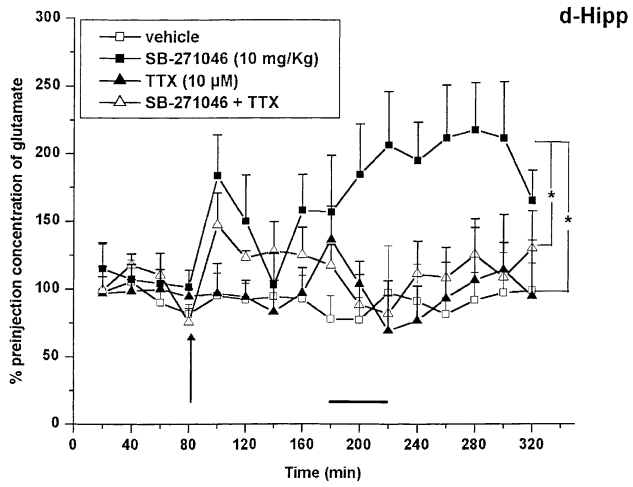

Figure 2. Effects of SB-271046 (10 mg/kg s.c) on extracellular levels of glutamate in the dorsal hippocampus of the freely moving rat. Data expressed as mean ± S.E.M, (n = 8–14 per study group). Arrow denotes subcutaneous drug or vehicle injection points; solid bar denotes TTX (10 μM) or vehicle infusion. * denotes statistical significance ($p < .05$) between groups.

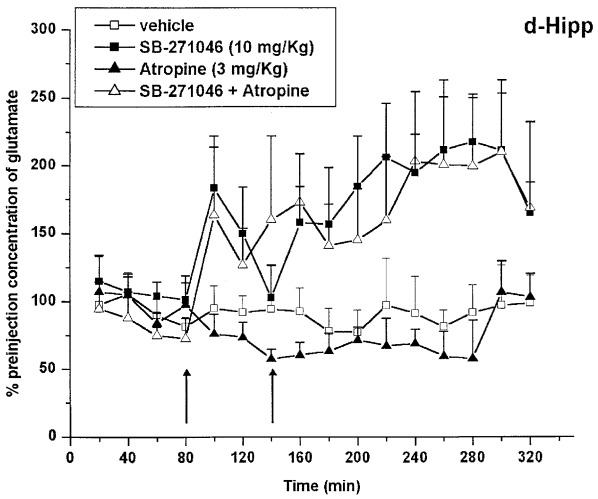

Figure 4. Effects of atropine (3 mg/kg s.c.) on SB-271046 (10 mg/kg s.c.) induced increases extracellular levels of glutamate within the dorsal hippocampus. Data expressed as mean ± S.E.M, (n = 8–14 per study group). Arrow denotes subcutaneous drug or vehicle injection points. * denotes statistical significance ($p < .05$) between groups.

dysfunction. However, very little neurochemical data has been reported to suggest the neurochemical mechanism behind these improvements in cognitive function. Using the selective antagonist SB-271046 (Bromidge et al. 1999) and *in vivo* microdialysis, we have examined the effects of 5-HT_6 receptor blockade on the release of monoamine/catacholamine and excitatory amino acid neurotransmitters in those brain regions demonstrated to have the highest 5-HT_6 receptor expression (Monsma et al. 1993; Ruat et al. 1993). SB-271046 produced no change in basal levels of either 5-HT, DA or NE in any brain region examined, indicating that under these experimental conditions the 5-HT_6 receptor is exerting no tonic control over the release of the conventional monoamine/catacholamine neurotransmitters. Alternatively, however, SB-271046 did produce significant 2- and 3-fold increases in the extracellular concentrations of the excitatory neurotransmitter glutamate, in both the hippocampus and frontal cortex, respectively.

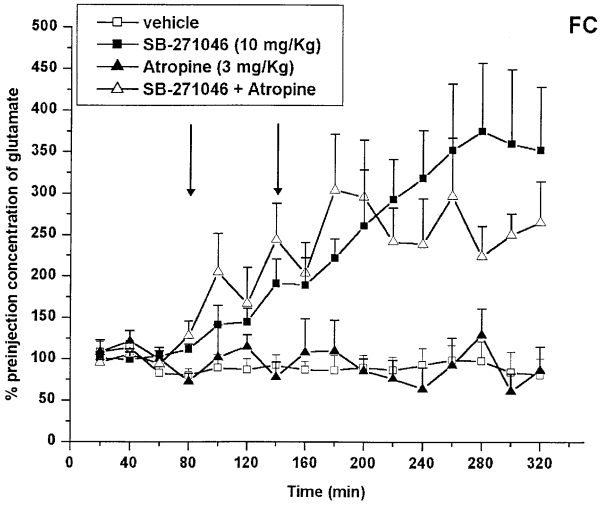

Figure 3. Effects of atropine (3 mg/kg s.c.) on SB-271046 (10 mg/kg s.c.) induced increases extracellular levels of glutamate within the frontal cortex. Data expressed as mean ± S.E.M, (n = 8–14 per study group). Arrow denotes subcutaneous drug or vehicle injection points. * denotes statistical significance ($p < .05$) between groups.

This is the first reported demonstration of the selective enhancement of excitatory neurotransmission by a 5-HT_6 antagonist within those brain regions which are thought to be critical in the control of cognitive and memory processes.

In order to confirm the neuronal origin of the 5-HT_6 receptor antagonist-induced increases in glutamate, we infused the voltage-dependent Na^+ channel blocker, tetrodotoxin (TTX), following SB-271046 administration. TTX infusion attenuated the SB-271046-induced increases in extracellular glutamate in both the frontal cortex and hippocampus, thus indicating that the increases in extracellular excitatory amino acid originate from glutamate neurons in an impulse dependent manner. It would therefore appear that there is a tonic serotonergic inhibition of glutamate neurons exerted either directly or indirectly via the 5-HT_6 receptor. At this time we can only speculate which glutamatergic projection systems are involved, based on reported 5-HT_6 localization (Monsma et al. 1993; Ruat et al. 1993). One possibility is that the enhanced transmission simply

originates from intrinsic glutamatergic neurons within both the cortex and hippocampus. Alternatively, the projection pathways of the basal ganglia/thalamocortical pathways may also be involved in those effects observed in the frontal cortex and perforant pathway and/or the amygdala/fornix connections may also be contributing to the effects in the hippocampus.

Since the 5-HT_6 receptor has been implicated in the modulation of cholinergic function (Bourson et al. 1995; Sleight et al. 1999; Bentley et al. 1999; Routledge et al. 1999) and the selective antagonist, Ro 65-7199, has been demonstrated to increase acetylcholine within both the cortex and hippocampus (Sleight et al. 1999), the influence of the cholinergic system on the observed glutamate effects was examined. Administration of muscarinic antagonist, atropine, at a dose that has previously been shown to block 5-HT_6 antisense oligonucleotide-induced behaviors, had no effect on the SB-271046-induced increases in extracellular glutamate in either brain region, thus indicating that the enhanced excitatory neurotransmission observed was not a consequence of an enhanced cholinergic function. Gerard et al. (1997) has suggested that 5-HT_6 receptors may be expressed on GABAergic spiny neurons and a very recent report (Woolley et al. 2000) actually showed co-localization of glutamic acid decarboxylase (GAD) immunoreactivity with 5-HT_6 receptors within multiple brain regions. Taken together, these data suggest that enhancement of glutamatergic function is not via a direct blockade of tonic serotonergic inhibition of glutamate neurons but is more likely to be an indirect action via the blockade of 5-HT_6 receptors on GABAergic interneurons either within, or on projection pathways to, the hippocampus and cortex. Whether the reported cholinergic effects (Sleight et al. 1999) are a consequence of this increase in glutamate or are an independent event (also mediated via GABA) cannot be determined from these experiments; however, interplay between the two systems has been demonstrated (Consolo et al. 1996; Sanz et al. 1997) and cannot be ruled out.

Interestingly, Routledge et al. (2000) has reported anticonvulsant properties of SB-271046 at doses much lower than those shown to be effective here and in other models (Rogers et al. 1999; Routledge et al. 1999; Dawson et al. 2000). The differences in effective dose may simply be due to the experimental paradigm employed. Electroconvulsive shock will not only increase the animal's serotonergic tone but also non-selectively stimulate multiple other transmitter systems. In contrast, microdialysis experiments are performed under resting conditions when there is little or no external stimulation; thus the endogenous serotonergic tone is likely to be much lower. However, the mechanism of this anticonvulsant action is not clear at this time, particularly, in light of enhanced basal glutamatergic neurotransmission and the hypothesized decrease in GABAergic input.

Since 5-HT_6 receptors are largely located in limbic regions and have nanomolar affinities for the atypical antipsychotic drugs, such as clozapine, it has been speculated that this receptor may have some involvement in schizophrenia. Deficits in glutamatergic function have been suggested to be causal in the cognitive and memory dysfunction observed in psychiatric patients (Hirsch et al. 1997; Breier 1999) and a number of atypical antipsychotics have been shown to be effective in the treatment of the cognitive deficits associated with schizophrenia (Tollefson 1996). A recent report by Healy and Meador-Woodruff (1999) provided direct evidence for a link between glutamatergic systems and 5-HT_6 receptors by showing that blockade of ionotropic glutamate receptors leads to a decrease in 5-HT_6 mRNA expression in various brain regions. Furthermore, evidence suggests that both frontal cortex and hippocampal structures are thought to play key roles in both cognition and memory function (Koechlin et al. 1999; Akhondzadeh 1999). Taken together, with our observations that 5-HT_6 receptor antagonists can enhance basal excitatory neurotransmission in both frontal cortex and hippocampus, it can be speculated that these types of compound will have therapeutic utility in the treatment of the cognitive and memory impairments associated with conditions such as schizophrenia.

In summary, we demonstrate for the first time the selective enhancement of excitatory neurotransmission, in both the frontal cortex and dorsal hippocampus, by the 5-HT_6 receptor antagonist SB-271046. These findings suggest a possible therapeutic role for 5-HT_6 receptor antagonists in the treatment of cognitive and memory dysfunction.

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