Acquisition, Retention, and Recall of Memory After Injection of RS67333, a 5-HT4 Receptor Agonist, Into the Nucleus Basalis Magnocellularis of the Rat

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The serotonin 5-HT4 subtype receptor is predominantly localized into anatomical structures linked to memory and cognition. A few experimental studies report that the acute systemic administration of selective 5-HT4 agonists has ameliorative effects on memory performance, and that these effects are reversed by contemporary administration of 5-HT4 receptor antagonists. To verify whether this procognitive action occurs via the activation of the cholinergic nucleus basalis magnocellularis (NBM)-cortical pathways, we examined the effects of RS67333, a selective partial agonist of the 5-HT4 receptor, on rat performance in a place recognition task upon local administration of the drug into the NBM area.

Intra-NBM administration of RS67333 enhances the acquisition (200–500 ng/0.5 µL) and the consolidation (40–200 ng/0.5 µL) of the place recognition memory. These effects are reversed by pretreatment with the selective 5-HT4 receptor antagonist RS39604 (300 ng/0.5 µL). Conversely, the recall of memory is not affected by the 5-HT4 agonist. Our results indicate that 5-HT4 receptors located within the NBM may play a role in spatial memory and that the procognitive effect of RS67333 is due, at least in part, to the potentiation of the activity of cholinergic NBM-cortical pathways.
RS67333 and Place Recognition Memory

RESULTS

Histology

Histological verification of injection sites of the animals that had completed the place recognition task was carried out by an observer blind to the behavioral results. A rat was classified as "good" when tips of both injection cannula tracks were within the area of the NBM and was classified as "lost" when one or both injection cannula tips were outside of the target area (Fig. 1B). All behavioral analyses reported in the present study were based on animals classified as "good."

Effects of Intra-NBM Injection of Saline, RS67333, and RS39604

The global analysis of data concerning saline-treated rats, operated and tested one week later (n = 29) indicated significant retention time (F 2/26 = 5.77; P = 0.008), arm (F 2/52 = 34.4; P < 0.001), and retention time × arm (F 4/52 = 8.3; P < 0.001) effects (Fig. 2). The number of visits to the novel arm was higher for the 60-min (F 2/16 = 14.5; P < 0.001; n = 9) and 120-min (F 2/18 = 68.9; P < 0.001; n = 10) groups but not for the 180-min interval group (F 2/18 = 0.04; P = 0.96; n = 10).

On the basis of these results, the drugs were tested for their promnestic or amnestic effects respectively at 180- or 120-min intertrial intervals. The 5-HT4 antagonist RS39604 (300 ng), infused preacquisition, postacquisition, and preretrieval into the NBM, produced no modifications of the rats’ performance at both 120-min (F 3/25 = 0.31; P = 0.82; n = 29) and 180-min (F 3/27 = 0.11; P = 0.96; n = 31) intertrial intervals. Conversely, the intra-NBM injection of RS67333 had ameliorative effects on the place recognition memory (Figs. 3, 4).

A significant effect of the treatment on rat performance was obtained after preacquisition (F 3/32 = 7.03; P = 0.001; n = 36) or postacquisition (F 3/34 = 11.02; P < 0.001; n = 38) administration of the 5-HT4 partial agonist. The preretrieval infusion of the drug was ineffective (F 2/23 = 0.68; P = 0.51; n = 26). A one-way ANOVA and Scheffe test indicated that the number of visits to the novel arm was higher in groups treated preacquisition with RS67333 200 ng (F 2/14 = 15.5; P < 0.001; n = 8) and 500 ng (F 2/16 = 11.3; P < 0.001; n = 9) or postacquisition with 40 and 200 ng (respectively: F 2/18 = 7.5; P = 0.004; n = 10 and F 2/16 = 10.6; P = 0.001; n = 9).

Time Course of RS67333 Postacquisition Effect

It can be seen in Figure 5 that the improvement of memory caused by the local infusion of 200 ng of RS67333 postacquisition was time-dependent. The overall ANOVA (n = 27) revealed significant delay (F 2/24 = 8.95; P = 0.0013), arm (F 2/48 = 20.52;
P < 0.001), and delay × arm (F 4/48 = 8.51; P < 0.001) effects. A 20-min delay did not affect the promnestic action of the drug (F 2/14 = 19.1; P < 0.001; n = 8), whereas the effect of the 5-HT4 agonist disappeared in the group injected 35 min after the end of first trial (F 2/18 = 2.05; P = 0.157; n = 10).

**DISCUSSION**

Several lines of evidence have suggested that there is a functional interaction between the cholinergic and serotonergic systems in the mediation of cognitive behavior (Sirvio et al. 1994; Cassel and Jeltsch 1995; Steckler and Sahgal 1995).

However, the initial studies reported some conflicting results about the role of the central 5-HT in learning and memory: These may be attributable to the application of a global strategy which modifies the whole serotonergic system and its interaction with other neurotransmitters, to the use of nonselective compounds, and to the systemic route of drug administration.

To better understand the relationships between the role of 5-HT in particular brain structures and defined cognitive functions, we investigated the modifications of spatial short-term memory caused by the local infusion (into the NBM) of two selective drugs, RS67333 and RS39604, that activate or inactivate a specific subtype (5-HT4) of 5-HT receptors.

Our results indicate that the local intra-NBM injection of RS67333 enhances the acquisition and the consolidation of place recognition memory. These effects were reversed by pretreatment with RS39604. Although the pharmacodynamic profile of RS67333 indicates that the compound has similar affinity for 5-HT4, α1, and α2 receptors (pKi = 8.7, 8.9, and 8.0, respectively; Eglen et al. 1995a), the antagonist RS39604 displays low affinity (pKi < 6.5) for 5-HT1A, 5-HT2C, 5-HT3, D1, D2, M1, M2, and µ receptors, low to moderate affinity for α1 (pKi = 6.8) and α2 (pKi = 7.8) receptors, and very high affinity for 5-HT4 sites (pKi = 9.1; Hedge et al. 1995). Given the selectivity of RS39604 for 5-HT4 receptors and the fact

**Figure 2** Place recognition memory (n = 29): The saline was injected into the NBM to three different groups of rats and the animals were tested in a Y-maze at increasing time intervals (60 min, n = 9; 120 min, n = 10; 180 min, n = 10) from the first trial (acquisition trial). The points indicate the number of visits for each arm (mean ± standard error of the mean [s.e.m.]). The statistical analysis of data (see the text for details) indicates that, in the present experimental conditions, the memory lasts about 120 min. *P < 0.05 compared with the other two arms (Scheffé post hoc test).

**Figure 3** Effect of RS67333 on the place recognition memory (n = 36): The intra-NBM injection of two increasing doses (200 ng/0.5 μL, n = 8; 500 ng/0.5 μL, n = 9) of the drug 2 min prior to the beginning of the first trial (preacquisition treatment) improves memory. A lower dose (40 ng/0.5μL, n = 10) has no effect. The points indicate the number of visits for each arm (mean ± s.e.m.). Intertrial interval, 180 min. *P < 0.05 compared with the other two arms (Scheffé post hoc test).
that in our experiments this compound reverses the memory improvement caused by RS67333 administration, a possible involvement of central 5-HT1 and 5-HT2 receptors in the observed behavioral effects of RS67333 may be rejected.

The present findings are supported by previous results of Fontana et al. (1997), who investigated the systemic action of RS67333 in a rat model of spatial memory, the Morris water maze. They observed a procognitive effect of the drug, attributable to central 5-HT4 activation, as it was abolished by pretreatment with RS39604, a selective receptor antagonist.

More recent studies add further evidence to the memory-enhancing properties of RS67333. Lelong et al. (2001) reported that the drug improved the learning rate of animals subjected to various training regimens in the Morris water maze. They observed a procognitive effect of the drug, attributable to central 5-HT4 activation, as it was abolished by pretreatment with RS67332, a selective receptor antagonist.

On the basis of the data reported here, it may be hypothesized that the ameliorative effect of RS67333 on place recognition memory, caused by 5-HT4 activation, results from the potentiation of the cholinergic NBM-cortical pathway. Other mixed 5-HT3 antagonists/5-HT4 agonists, the benzimidazolones BIMU-1 and BIMU-8, in fact, increased extracellular levels of acetylcholine (ACh) in rat microdialysis studies (Consolo et al. 1994), and BIMU-8 enhanced the ACh efflux in electrically stimulated slices of cerebral cortex, hippocampus, and NBM (Siniscalchi et al. 1999). In addition, the administration of RS67333 reversed the cognitive deficit induced by atropine, a nonselective muscarinic antagonist, in the Morris water maze (Fontana et al. 1997).

The cholinergic input to the neocortical mantle is believed to be of crucial importance in cortical functions. In addition, the degeneration of cholinergic neurons of the basal forebrain and the loss of presynaptic cortical cholinergic markers are assoc-

<Figure 4> Effect of RS67333 on the place recognition memory (n = 38): The intra-NBM injection of two increasing doses (40 ng/0.5 µL, n = 10; 200 ng/0.5 µL, n = 9) of the drug within 30 sec from the end of the first trial (postacquisition treatment) improves memory. A higher dose (500 ng/0.5 µL, n = 12) has no effect. The points indicate the number of visits for each arm (mean ± s.e.m.). Intertrial interval, 180 min. *P < 0.05 compared with the other two arms (Scheffé post hoc test).

<Figure 5> Time-dependency of the ameliorative effect of RS67333 (n = 27) on place recognition memory: The same dose (200 ng/0.5 µL) of the drug was injected into the NBM 30 sec (n = 9), 20 min (n = 8), or 35 min (n = 10) after the end of the acquisition trial to three different groups of rats. The statistical analysis of data (see the text for details) indicates that the procognitive effect of RS67333 disappears when the drug is injected with a 35-min delay. The points indicate the number of visits for each arm (mean ± s.e.m.). Intertrial interval, 180 min. *P < 0.05 compared with the other two arms (Scheffé post hoc test).

Gasbarri et al. (1999) revealed that serotonergic terminals, deriving primarily from the B7 cell group of the dorsal raphe nucleus, make putative synaptic contacts with cholinergic neurons of the NBM. Those authors thus provided neuroanatomical evidence of a direct functional interaction between the two neurotransmitter systems in the basal forebrain. Our results support these findings, indicating the presence and the prominent role of the 5-HT4 receptors within this brain area.

In the present study the preacquisition, postacquisition, and preretrieval administration of 300 ng of the 5-HT4 antagonist RS39604 into the NBM did not affect memory by itself, being able to reverse the procognitive effect of RS67333. This finding indicates that, in our experimental conditions, the behavioral performance is associated, at the level of the NBM, with a low release of endogenous 5-HT. Therefore the finding demonstrates that the effect of the partial agonist RS67333 is attributable to the activation of 5-HT4 receptors rather than to the blockade of the effects of the full agonist 5-HT.

Figure 5 Time-dependency of the ameliorative effect of RS67333 (n = 27) on place recognition memory: The same dose (200 ng/0.5 µL) of the drug was injected into the NBM 30 sec (n = 9), 20 min (n = 8), or 35 min (n = 10) after the end of the acquisition trial to three different groups of rats. The statistical analysis of data (see the text for details) indicates that the procognitive effect of RS67333 disappears when the drug is injected with a 35-min delay. The points indicate the number of visits for each arm (mean ± s.e.m.). Intertrial interval, 180 min. *P < 0.05 compared with the other two arms (Scheffé post hoc test).
associated with the cognitive decline in patients with Alzheimer's disease. In this respect, the potentiating action of 5-HT4-selective agonists on cholinergic function and their memory-enhancing effects might have significant implications for the development of novel approaches to the treatment of cognitive disorders.

Our data also indicate that the procognitive effect of RS67333 appears when the drug is injected before and immediately after the first trial of the place recognition task. Thus, the activation of 5-HT4 receptors facilitates the acquisition and the consolidation of spatial information. In contrast, the memory recall is not affected by the 5-HT4 agonist.

Interestingly, the postacquisition effect of RS67333 on place recognition was time-dependent, because the promnesic action of RS67333 disappeared when the intra-NBM infusion of the drug was made 35 min after the end of the first trial. These results indicate that, in the NBM, the biochemical events triggered by 5-HT4 receptors activation could play a role in the early period of memory consolidation. In this respect, the observation that the immediate postacquisition phase (<30 min after training) is also subjected to the modulatory effects of central muscarinic and nicotinic synapses (Izquierdo and Medina 1997) is in line with the hypothesis of cholinergic-mediated effects of 5-HT4 receptor agonists on memory.

Although the present findings support and extend those reported by other workers, they are in contrast to the results of Meneses and Hong (1997). Those authors reported that the pretraining activation of 5-HT4 receptors enhanced the acquisition, whereas the postraining activation of 5-HT4 receptors impaired the consolidation, of an appetitive lever-press response in rats treated with BIMU 1 and BIMU 8.

The apparent discrepancy between the data of Meneses and Hong and the present results can be easily explained by taking into account differences in behavioral task (operant task vs. spatial, short-term memory task) and experimental protocol employed (repeated training vs. single training trial, 24-h retention interval vs. 180-min retention interval, intra-NBM drug infusion vs. systemic administration). Moreover, our postacquisition data are in agreement with those reported by Galeotti et al. (1998), who observed, in a mouse passive avoidance test, a facilitation of memory after postraining administration of BIMU 1 and BIMU 8, two mixed 5-HT3 antagonists/5-HT4 agonists.

In conclusion, the experiments reported here demonstrate that: (1) The activation of 5-HT4 receptors enhances the acquisition and the consolidation but not the recall of spatial memory; (2) 5-HT4 receptors are located within the NBM and may play a role in learning and memory; and (3) the procognitive effect of RS67333 is likely due, at least in part, to the potentiation of the activity of cholinergic NBM-cortical pathways.

Taken together, these findings seem to indicate that the administration of selective 5-HT4 receptor agonists in conjunction with acetylcholinesterase inhibitors may provide a more effective treatment strategy for age-related cognitive impairments compared to therapies designed to potentiate selectively the cholinergic synaptic transmission. Moreover, a recent study by Lamiu et al. (2003) regarding the effects on place and object recognition memory of a combined treatment with RS67333 and galantaminium bromide, a new cholinesterase inhibitor, strongly supports this hypothesis.
MATERIALS AND METHODS

Rats

Male Wistar rats (Charles River) weighing 180–200 g were used as subjects. They were housed in a temperature-controlled colony room (21 °C ± 1°C) with free access to food and water and were maintained four per cage under standard laboratory conditions. Before surgery, each rat was submitted to daily handling for at least 1 wk. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Surgery

The rats were anesthetized with Domitor (0.5 mg/kg) plus ketamine (75 mg/kg) i.p., placed in a stereotaxic apparatus (Stoelting, Stellar), and implanted bilaterally with chronic indwelling guide cannulae (23-gauge) positioned 1 mm above the final injection sites. The coordinates were AP = −1.3, L = ±2.8, and V = −3.6 relative to bregma (Paxinos and Watson 1998). The guide cannulae were held in place with stainless steel screws and dental acrylic cement and were occluded with removable stainless steel wire pins. Postoperatively, the animals were given free access to food and water and were allowed to recover for 1 wk before behavioral testing.

Apparatus and Behavioral Task

The apparatus used was a Y-maze made of opaque Plexiglas. The maze was placed in a sound-isolated room equipped with constant illumination (a 60-W lamp located 150 cm above the center of the maze). Several visual cues were placed in the testing room near the maze and were kept constant during all the experiments. The test of place recognition (Dellu et al. 1992) consisted of two trials, separated by different retention intervals (in saline experiments) or by a fixed retention interval (in drug experiments). Each rat was subjected to this behavioral paradigm only once. In the first trial (acquisition trial), one arm of the maze was closed with a guillotine door, and the rat was allowed to visit the other two open arms for 10 min. During the second trial (testing trial), the rat had free access to the three arms and was allowed to explore the maze for 5 min. At the beginning of both trials, each rat was placed in the same arm (the entry arm) and was oriented in the direction opposite of the center of the maze. In our experiments, the entry arm was changed randomly to reduce the influence of individual external cues on animal performance. Similarly, the position of the novel arm (the arm closed by the guillotine door in the acquisition trial) was kept in a random order at the left for half the rats and at the right of the entry arm for the other half. During the testing trial, the numbers of visits of the novel, familiar, and entry arms were recorded separately, and the values so obtained are expressed as means ± SEM. Recognition was assumed to have occurred when the rat made more visits to the novel arm than the other two familiar arms. After each trial, the maze was carefully cleaned to eliminate olfactory stimuli.

Drugs and Injection Procedure

RS67333 hydrochloride and RS39604 hydrochloride (Tocris Cookson) were dissolved in saline (NaCl 0.9% solution) and prepared immediately prior to use. The control group rats received only the saline. Intracranial administration was made by two infusion pumps (CMA model 102) connected to 28-gauge injection cannulae with polyethylene tubing. The injection cannulae were inserted to a depth of 1 mm below the tips of the guide cannulae, and the solutions were infused at a rate of 2 µl/min for 15 sec. The injection cannulae were left in place for an additional 30-sec period to promote diffusion.

In control experiments, three groups of saline-treated rats were tested at increasing time intervals (60, 120, and 180 min) from the acquisition trial. Within each group, the saline was administered randomly preacquisition, postacquisition, or pre-retrieval, because preliminary experiments indicated no differences among these treatments. The drugs were administered 2 min prior to the beginning of the first trial (preacquisition treatment) or the second trial (preretrieval treatment) or within 30 sec from the end of the first trial (postacquisition treatment).

To estimate the range of drug diffusion following local infusion, blue dye (Fast Blue, Dupont) was dissolved in 0.9% saline at a concentration of 25 µg/0.25 µl and infused at the same rate as RS67333 and RS39604 (Fig. 1).

Historical

At the conclusion of testing, all operated animals were anesthetized and the brains were perfused with 10% formalin-saline solution. The brains were removed, postfixed in 30% formalin for 48 h, and sectioned into 80-µm sections. Finally the sections were stained with cresyl-violet for histological verification of the injection sites (Fig. 1).

Data Analysis

The global analysis of experimental data was performed by a mixed design between-within ANOVA. Within each group, the presence of a significant arm effect, that is, the rats’ for a particular arm, was evaluated by one-way ANOVA with repeated measures followed, when appropriate, by post hoc two-by-two comparisons (Schefфе test). The significance threshold was P = 0.05.

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