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The Selective 5-HT$_6$ Receptor Antagonist Ro4368554 Restores Memory Performance in Cholinergic and Serotonergic Models of Memory Deficiency in the Rat

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Antagonists at serotonin type 6 (5-HT$_6$) receptors show activity in models of learning and memory. Although the underlying mechanism(s) are not well understood, these effects may involve an increase in acetylcholine (ACh) levels. The present study sought to characterize the cognitive-enhancing effects of the 5-HT$_6$ antagonist Ro4368554 (3-benzenesulfonyl-7-(4-methyl-piperazin-1-yl)1H-indole) in a rat object recognition task employing a cholinergic (scopolamine pretreatment) and a serotonergic- (tryptophan (TRP) depletion) deficient model, and compared its pattern of action with that of the acetylcholinesterase inhibitor metrifonate. Initial testing in a time-dependent forgetting task employing a 24-h delay between training and testing showed that metrifonate improved object recognition (at 10 and 30 mg/kg, p.o.), whereas Ro4368554 was inactive. Both, Ro4368554 (3 and 10 mg/kg, intraperitoneally (i.p.)) and metrifonate (10 mg/kg, p.o., respectively) reversed memory deficits induced by scopolamine and TRP depletion (10 mg/kg, i.p., and 3 mg/kg, p.o., respectively). In conclusion, although Ro4368554 did not improve a time-related retention deficit, it reversed a cholinergic and a serotonergic memory deficit, suggesting that both mechanisms may be involved in the facilitation of object memory by Ro4368554 and, possibly, other 5-HT$_6$ receptor antagonists.
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INTRODUCTION

The use of selective serotonin (5-hydroxytryptamine; 5-HT) agonists and antagonists has increased our understanding of the role of 5-HT receptors in processes of learning and memory (Barnes *et al*, 1992; Buhot *et al*, 2000). Recently, serotonin type 6 (5-HT$_6$) receptors were shown to be targets for cognitive-enhancing drugs (Meneses, 1999).

The 5-HT$_6$ receptor was first identified in 1993 and is localized in many brain areas. These include brain structures associated with learning and memory processes such as the hippocampus and the cerebral cortex (Gerard *et al*, 1997; Roberts *et al*, 2002; Ruat *et al*, 1993). Antagonism of 5-HT$_6$ receptors leads to an increase in the release of acetylcholine (Ach) (Riemer *et al*, 2003; Shirazi-Southall *et al*, 2002), but whether this is directly caused by antagonism at these receptors is still under debate. Some evidence suggests that the cholinergic system might be activated indirectly through an increase in the excitatory amino acids aspartate and glutamate (Bourson *et al*, 1998; Dawson *et al*, 2000).

SB271046 (Bromidge *et al*, 1999) and Ro046790 (Sleight *et al*, 1998) were the first 5-HT$_6$ antagonists to be evaluated for their behavioral effects, and both compounds improved retention in a Morris water maze task in rats, suggesting an improvement of spatial memory (Rogers and Hagan, 2001; Woolley *et al*, 2001). Ro046790 enhanced nonspatial working memory in an object recognition task (ORT) (Woolley *et al*, 2003), increased consolidation in an autoshaping task, and reversed scopolamine-induced deficits in both an autoshaping and a passive avoidance task (Bos *et al*, 2001; Meneses, 2001). However, some of these effects in the autoshaping and Morris water maze task could not be replicated (Lindner *et al*, 2003; Russell and Dias, 2002). Although the reason is not known, poor drug properties, such as a low affinity for the 5-HT$_6$ receptor and a low penetration of the blood–brain barrier, may contribute to a higher variability in behavioral findings with both compounds.

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Recently, 5HT$_6$ receptor antagonists with improved affinity and selectivity have been developed that demonstrate better penetration into the central nervous system (Russell and Dias, 2002). One of these drugs is Ro4368554, which possesses a blood–brain penetration of 80–110% (against 1 and 10% for Ro046790 and SB271046, respectively). *Ex vivo* findings confirmed the good brain penetration of Ro4368554, showing 50% occupancy of brain 5-HT$_6$ receptors at 7.8 mg/kg, intraperitoneally (i.p.) (Bonhaus *et al*, 2002). Hence, a first objective of the present study was to test whether Ro4368554 would possess procognitive activity in a rat model for recognition memory: the novel ORT (Ennaceur and Delacour, 1988). The initial characterization of Ro4368554 in models for learning and memory has been published in an abstract (Szczepanski *et al*, 2002).

A second aim of our study was to compare the potential cognitive-enhancing effects of Ro4368554 with those of metrifonate in unimpaired animals as well as against a scopolamine deficit in the novel ORT. Metrifonate is a acetylcholinesterase inhibitor (AChEI) and increases ACh availability in the synapse in a more direct manner than 5-HT$_6$ antagonists (Cummings *et al*, 1998; Ringman and Cummings, 1999). Preclinical studies demonstrated that metrifonate improved age-impaired memory functions (spatial and nonspatial) (Dachir *et al*, 1997; Scali *et al*, 1997; van der Staay *et al*, 1996a) and scopolamine-induced impairments in water maze and passive avoidance tasks (Itoh *et al*, 1997; Riekkinen *et al*, 1996). Although treatment with metrifonate can stabilize cognitive functions in Alzheimer patients for at least 6 months (Becker *et al*, 1998), clinical development was discontinued in 2000 since some patients experienced muscle weakness in Phase III trials. We selected metrifonate for our study since it shows a significantly better tolerability profile in rodents than other AChEIs such as Aricept (Blokland *et al*, 1995; van der Staay *et al*, 1996b).

Other transmitter systems, such as the noradrenergic, dopaminergic, and serotonergic system, are also involved in learning and memory processes (Blokland, 1995; Myhrer, 2003; Robbins, 1998). The serotonergic system is believed to be especially relevant in mediating the acquisition processes in memory formation (Meneses, 1999; Myhrer, 2003), although its precise function is not fully known and studies have sometimes led to controversial findings (McEntee and Crook, 1991). Dysfunctional memory occurred after acute lowering in plasma tryptophan (TRP; precursor of 5-HT) levels (Riedel *et al*, 2002). The cognitive impairments after TRP depletion were found in healthy volunteers (Park *et al*, 1994; Riedel *et al*, 1999; Rubinsztein *et al*, 2001), as well as in patients with neurological disorders such as Alzheimer disease (Porter *et al*, 2003), bipolar disorders (Sobczak *et al*, 2002) or schizophrenia (Golightly *et al*, 2001).

We found that an acute and substantial reduction ($\sim$65%) in plasma TRP levels impaired performance in an ORT several hours after adult rats were given a TRP-free, protein–carbohydrate mixture (Lieben *et al*, 2004b). These effects appeared to be selectively associated with cognitive function since anxiety- and depressive-like behavior were not affected. These data suggest that this method of acute TRP depletion could be used as model of dysfunction in object recognition. Therefore, a third aim of the present study was to test Ro4368554 and metrifonate in a TRP depletion model of cognitive function and to compare their effects with those obtained in the time-related forgetting and scopolamine-deficit studies.

MATERIALS AND METHODS

Animals

All experiments were conducted as described in protocols approved by the Animal Research Committee. In general, the experiments were carried out with groups of 24, 4-month-old male Wistar rats (Charles River), except for the scopolamine (dose–response study, $n=18$) and the metrifonate/TRP study ($n=12$). Rats were individually housed in standard Makrolon® cages on sawdust bedding in an air-conditioned room ($\pm$20°C). Rats had free access to food and water and were kept under a reversed 12/12 h light/dark cycle (lights off from 0600 to 1800 h).

Drugs and Chemicals

Scopolamine hydrobromide and L-TRP were obtained from Sigma (Zwijndrecht, The Netherlands and CA, USA, respectively). Metrifonate was kindly donated by Bayer AG (Wuppertal, Germany). The selective 5-HT$_6$ antagonist Ro4368554 (3-benzenesulfonyl-7-(4-methyl-piperazin-1-yl)1H-indole) was provided by Roche Pharmaceuticals (Palo Alto, CA, USA). The gelatin hydrolysate (Solugel C®) was purchased from PB Gelatins (Tessenderlo, Belgium). Maltodextrine was purchased from the Amylumgroup (Koog aan de Zaan, The Netherlands).

Treatment

An overview of the different experiments is given in Table 1. Over a period of 2 weeks preceding each experiment, the rats were handled and habituated to injections with saline (NaCl, 0.9%; 1 ml/kg). On days of administration, Ro4368554 was dissolved in saline. Administration was always given i.p. (injection volume 1 ml/kg) 60 min before trial 1 (T1). Ro4368554 was tested at doses of 1, 3, and 10 mg/kg. Testing order of doses was determined randomly and balanced across test days. All rats were treated once with each dose condition. Metrifonate was dissolved in 0.1 M sodium citrate buffer (pH 5.5), to prevent the nonenzymatic transformation into dichlorvos in solution, on every experimental day. It was administered, p.o. in an injection volume of 1 ml/kg, 30 min before behavioral testing. Metrifonate was tested at three different doses (3, 10, and 30 mg/kg, p.o.), except for the acute TRP depletion experiment, where metrifonate was injected at 1, 3, and 10 mg/kg. Scopolamine was dissolved in saline and injected subcutaneously (s.c.) (2 ml/kg) or i.p. (1 ml/kg). The administration of scopolamine was 30 min before testing. First, a dose–response curve of scopolamine was established (0.03, 0.1, and 0.3 mg/kg). The minimal effective dose of scopolamine was used for further drug testing.

In the TRP depletion experiment, the animals were handled and habituated to oral injections with normal tap water (10 ml/kg) and with the protein–carbohydrate mixture enriched of TRP. In the experimental condition, food was removed 14 h before the treatment to minimize TRP uptake from food. At all times, the animals had free access

Table 1 Overview of the Different Behavioral Experiments

	Baseline	**Ro4368554**	**Metrifonate**
24-h delay	1 *vs* 24 h $n = 24$	0/1/3/10 mg/kg i.p.: 1 ml/kg 60 min before T1 $n = 24$	0/3/10/ 30 mg/kg i.p.: 1 ml/kg 30 min before T1 $n = 24$
Scopolamine	0/0.03/0.1/0.3 mg/kg s.c.: 2 ml/kg 30 min before T1 $n = 24$ 0/0.03/0.1/0.3 mg/kg i.p.: 1 ml/kg 30 min before T1 $n = 18$	Scop–vehicle/0/1/3/10 mg/kg Scopolamine: 0.1 mg/kg i.p.: 1 ml/kg 60 min before T1 $n = 24$	Scop–vehicle/0/3/10/30 mg/kg Scopolamine: 0.1 mg/kg i.p.: 1 ml/kg 30 min before T1 $n = 24$
Acute TRP depletion	TRP+ *vs* TRP− p.o.: 10 ml/kg 240 and 150 min before T1 $n = 24$	TRP+/0/1/3/10 mg/kg TRP− i.p.: 1 ml/kg 60 min before T1 $n = 24$	TRP+/0/1/3/10 mg/kg TRP− i.p.: 1 ml/kg 30 min before T1 $n = 12$

Columns indicate the treatment that was given. The rows indicate the model used to induce a deficit in object recognition. For each experiment, the doses used, the route, and injection volume is given, together with the time of injection, and the number of animals.

to water. The rats were treated either with a TRP lacking protein–carbohydrate mixture (TRP−) or an identical mixture containing an additional amount of TRP (TRP+). The composition of the meal is described elsewhere (Lieben *et al*, 2004b). On the experimental day, the animals were injected twice before the first trial of the ORT (T1), using an interval of 90 min. The first administration was given 4 h before T1 and the second injection 2.5 h before T1. Each administration consisted of 4 g Solugel C and 2 g Malthodextrine in a volume of 10 ml/kg, p.o.

Object Recognition Task

The ORT was performed as described in detail elsewhere (Ennaceur and Delacour, 1988; Lieben *et al*, 2004b). In short, the apparatus consisted of a circular arena, 83 cm in diameter. Half of the 40-cm-high wall was made of gray polyvinyl chloride, the other half of transparent polyvinyl chloride. The light intensity ($\sim$20 Lx) was equal in the different parts of the apparatus. Two objects were placed in a symmetrical position about 10 cm away from the gray wall. We used four different sets of objects that could not be displaced by a rat. The different objects used were as follows: (1) a cone consisted of a gray polyvinyl chloride base (maximal diameter 18 cm) with collar on top made of brass (total height 16 cm), (2) a 1-l transparent glass bottle (diameter 10 cm, height 22 cm) filled with water, (3) a massive metal cube ($10 \times 5 \times 7.5$ cm) with two holes (diameter 1.9 cm), and (4) a massive aluminum cube with a tapering top ($13 \times 8 \times 8$ cm). Each object was available in triplicate.

In the first week, the animals were adapted to the procedure, that is, they were allowed to explore the apparatus (without any objects) twice for 3 min each day. In the two following weeks, the rats were trained until they showed a stable discrimination performance (ie either a good object discrimination at a 1-h interval or a lack of recognition at a 24-h interval). Subsequently, drug testing began.

A testing session comprised of two trials. The duration of each trial was 3 min. During the first trial (T1), the apparatus contained two identical objects. A rat was always placed in the apparatus facing the wall at the middle of the front (transparent) segment. After the first exploration period, the rat was put back in its home cage. After a delay interval (1 or 24 h), the rat was put back in the apparatus for the second trial (T2), but now with dissimilar objects, a familiar one and a novel one. The duration of exploring each object at T1 and T2 was recorded manually with a personal computer. Exploration was defined as follows: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered as exploratory behavior. In order to avoid the presence of olfactory trails, the objects were always thoroughly cleaned between trials of individual animals. Moreover, each object was available in triplicate so none of the two objects from the first trial had to be used as the familiar object in the second trial. In addition, all combinations and locations of objects were used in a balanced manner to reduce potential biases due to preferences for particular locations or objects.

Performance at baseline level was assessed using a 1-h delay, that is, a time interval where object discrimination is expected, and a 24-h delay, that is, a time interval where Wistar rats no longer can discriminate the novel object from the object that they previously explored (eg Prickaerts *et al*, in press). When using a 24-h delay, two sessions were given every week, one session comprised Monday and Tuesday and the other one comprised Thursday and Friday. Since Ro4368554 and metrifonate were expected to improve baseline memory performance, we used a 24-h delay at which no discrimination between the objects occurs. In contrast, a 1-h delay was used for drug testing in animals treated with scopolamine and the protein–carbohydrate mixture.

Statistical Analysis

The basic measures were the total exploration time of both objects during T1 and T2, *e*1 and *e*2 (total exploration to both objects in trial 1 and trial 2, respectively). The discrimination index ($d2 =$ (exploration novel object− exploration familiar object)/*e*2) is a relative measure of discrimination that corrects for the total exploration activity in the second trial.

Since no difference in performance was observed between similar treatment condition (comparisons within conditions tested with a *t*-test: *t*'s < 0.85, NS), the data of the *d*2 values were pooled by dose condition across the sessions. Subsequently, for each subject the mean of each variable was calculated. Furthermore, the data of both the vehicle–vehicle condition and the scopolamine–vehicle condition were pooled over the metrifonate and the Ro4368554 study. The same procedure was applied for the TRP+ and the TRP− conditions.

Overall comparisons of exploration times (*e*1 and *e*2) between the different dose conditions of each treatment were analyzed with a one-factorial ANOVA. Apparent effects were evaluated in more detail with a Dunnett *post hoc* analysis (two-sided, $p < 0.05$) by comparing the results of each condition to those of a control group (eg vehicle, scopolamine, or TRP+ group).

To evaluate the discrimination performance, we compared the *d*2 values between the different treatment conditions using an ANOVA with a Dunnet *post hoc* test. In addition, we compared the *d*2 values of the different doses with a virtual group, which was formed on the basis of seven vehicle sessions from previous experiments in which animals were tested on a 24-h delay interval, that is, a condition where there is no recognition (see Şık *et al*, 2003). This virtual group had a mean *d*2 of zero and an SEM of 0.07. Comparisons with these groups evaluate more reliably whether discrimination performance differs from zero in a certain test condition. In addition, natural fluctuation in control conditions (in this case 24-h delay, scopolamine, and TRP−) may be either slightly higher or lower than zero. This may lead to underestimation or overestimation of drug effects, respectively. Comparisons between the *d*2 of the different dose conditions and the virtual group were assessed by an analysis of variance (ANOVA). In case of a statistically reliable dose effect, comparisons between the different doses and the virtual group were analyzed in more detail using a one-sided Dunnett *post hoc* test. Since by definition the memory performance cannot be negative ($d2 < 0$), a one-sided test was used. Thus, no memory means $d2 = 0$, whereas memory for objects represents a $d2 > 0$.

To examine to possible differences in the route of scopolamine administration (i.p. or s.c.) on discrimination performance, the data of scopolamine treatment were analyzed with a two-factorial (Injection by Dose) ANOVA with the factor Dose as repeated measures. Since the effect of injection was tested in two different set of experiments, Injection was used as a between-subjects factor.

The effects of Ro4368554 and metrifonate were tested within the three testing conditions (time, scopolamine, and TRP depletion), separately. In each testing condition, the effects of the different doses of Ro4368554 and metrifonate were tested in the same animals. Consequently, Dose was used as a repeated measures factor. The effects of Ro4368554 and metrifonate were not evaluated between different testing conditions.

RESULTS

Baseline Performance

Time-dependent forgetting. In the first trial of the 1-h interval condition, rats spent a total time of 21 (20.15) s (mean (SEM)) exploring both identical objects, while in the second trial, they explored the objects over a period of 28 (2.0) s. In the 24-h delay condition, the animals explored the objects for 26 (1.6) and 30 (2.0) s during T1 and T2, respectively. The exploration times in both trials did not differ between the 1- and 24-h conditions (t_{46}'s, NS). However, an increase in activity from T1 to T2 was found in both the 1- and 24-h delay condition (t_{46}'s > 3.10, $p < 0.01$).

The discrimination index at the 1-h delay differed from the virtual group ($d2 = 0.36$ (0.03); $t_{46} = 3.54$, $p < 0.001$), indicating that the rats spent more time investigating the novel object. However, the discrimination index was low after a 24-h delay ($d2 = 0.05$ (0.05)), and the *d*2 value was not different from the virtual group ($t(46) = 0.34$, NS).

Scopolamine. Scopolamine did not affect the exploration time in T1 (Dose: $F_{3,120} = 0.46$, NS; see Table 2) and was similar for both routes of administration (Injection: $F_{1,40} = 0.02$, NS). Scopolamine affected the activity in T2 (Dose: $F_{3,120} = 4.57$; $p < 0.01$). Although the effects on exploration time in T2 were dependent on the injection method used ($F_{1,40} = 8.62$, $p < 0.01$), no interaction occurred between the two factors (Injection × Dose: $F_{3,120} = 0.45$, NS). A dose effect was found on the relative discrimination index after treatment with scopolamine ($F_{3,120} = 3.49$, $p < 0.05$; see Figure 1). In contrast to the vehicle condition, rats treated with scopolamine at a dose of 0.1 and 0.3 mg/kg did not discriminate between the objects: for both doses, the *d*2 values were not different from those of the virtual control group. Furthermore, the effects on the relative discrimination index did not depend on the route of injection ($F_{1,40} = 0.02$, NS) and no interaction effect was found (Injection × Dose: $F_{3,120} = 0.36$, NS). The minimal effective dose was 0.1 mg/kg s.c. (see Figure 1). Since the route of administration did not affect the memory performance, the i.p. and s.c. data were pooled for the evaluation of drug effects.

Table 2 Effects of Scopolamine on Exploration Times in the ORT Using a 1-h Interval

Scopolamine (s.c. 2 ml/kg)			Scopolamine (i.p. 1 ml/kg)		
Dose (mg/kg)	**e1**	**e2**	**Dose (mg/kg)**	**e1**	**e2**
0	30 (2.2)	30 (1.9)	0	26 (2.1)	34 (2.1)
0.03	28 (2.0)	23 (1.9)	0.03	29 (2.0)	31 (2.4)
0.1	24 (2.0)	28 (2.4)	0.1	30 (2.2)	34 (1.7)
0.3	31 (2.5)	33 (2.7)	0.3	28 (2.7)	37 (2.8)

Rats received injections of scopolamine hydrobromide at doses of 0.03, 0.1, and 0.3 mg/kg, 30 min before T1. Scopolamine injection was given s.c. at a volume of 2 ml/kg or i.p. at a volume of 1 ml/kg. Mean values (±SEM) of total exploration time (s) during the first (e1) and the second trial (e2) are presented. Differences from vehicle condition: $p<0.05$.

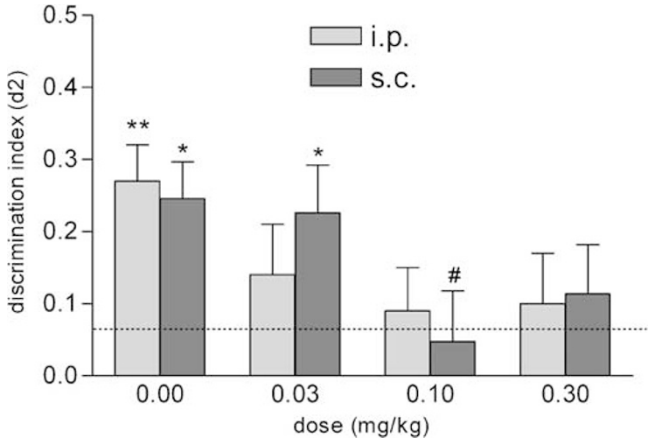

Figure 1 Effects of scopolamine-Hbr (hydrobromide) on the discrimination performance in an ORT in adult male rats using a 1-h delay (mean + SEM). In the vehicle session, rats were treated with saline. In the drug session, rats were treated with doses of 0.03, 0.1, and 0.3 mg/kg, s.c. 2 ml/kg and i.p. 1 ml/kg. Different from virtual control group (indicated by the dotted line): $^{*}p<0.05$; $^{**}p<0.01$. Differences from vehicle group: $^{\#}p<0.05$.

Acute TRP depletion. In both trials (T1 and T2), no differences in exploration times were found between the TRP+ (35 (2.5) and 44 (3.5) s, respectively) and the TRP− (38 (2.6) and 46 (2.9) s, respectively) group (t_{44}'s, NS). A treatment effect was found on the relative discrimination index ($t_{44}=2.52$, $p<0.01$). While in the TRP+ condition, the rats could discriminate between objects in the second trial ($d2=0.37$ (0.04); significantly different from virtual control group, $p<0.001$), the $d2$ value was reduced to chance level in the TRP− condition ($d2=0.07$ (0.04)) and *post hoc analysis* did not reveal a difference between the TRP− and the virtual group.

Effects of Ro4368554

Time-dependent forgetting. Exploration levels in T1 were affected by treatment with Ro4368554 ($F_{3,85}=6.20$, $p<0.05$; see Table 3a), although *post hoc* analysis did not reveal differences between Ro4368554-treated groups and the vehicle-treated group. The exploration times in T2 were not affected by treatment with Ro4368554 ($F_{3,85}=0.43$, NS). After treatment with Ro4368554, the rats did not discriminate between the novel object and the familiar object during T2 ($F_{3,85}=0.74$, NS; see Figure 2).

Scopolamine. In both trials, explorative behavior was affected by combined treatment with scopolamine and Ro4368554 (F's > 5.09, $p<0.01$; see Table 3b). As compared with the vehicle-treated group, *e*1 values were lower in the group treated with 1 mg/kg Ro4368554 and higher in the group treated with 3 mg/kg Ro4368554. Treatment with Ro4368554 reversed the scopolamine-induced object discrimination deficit ($F4,160=5.62$, $p<0.01$; see Figure 3). *Post hoc* analysis revealed that the $d2$ value of the 3 and 10 mg/kg was different from both the scopolamine-treated and the virtual group.

Acute TRP depletion. No treatment effects in explorative behavior were found in T1 and T2 ($F_{4,126}$'s < 2.28, NS; see Table 3c). Treatment with Ro4368554 reversed the acute TRP depletion-induced deficit on the discrimination performance ($F_{4,126}=7.22$, $p<0.01$). Figure 4 shows no effects of 1 and 3 mg/kg Ro4368554, and the discrimination index did not differ from the virtual group. Treatment with 10 mg/kg Ro4368554 improved object recognition in the TRP− condition as compared with the vehicle and virtual group.

Effects of Metrifonate

Time-dependent forgetting. The exploration times for *e*1 and *e*2 were not affected by treatment with metrifonate ($F_{3,91}$'s < 1.32, NS; see Table 3a), but explorative behavior tended to be higher for *e*2. Treatment with metrifonate improved object discrimination at a 24-h retention interval ($F_{3,91}=2.96$, $p<0.05$; see Figure 2). *Post hoc* analysis showed that 10 and 30 mg/kg metrifonate improved the memory performance when compared with the virtual group.

Scopolamine. Exploration time in T1 was affected by treatment ($F_{4,16}=3.95$, $p<0.01$; see Table 3b). *Post hoc* analysis showed that rats were more active in the group treated with 30 mg/kg metrifonate. No treatment effect on explorative behavior in T2 was found ($F_{4,162}=0.88$, NS). The $d2$ value was affected by metrifonate ($F_{4,162}=5.37$, $p<0.01$; see Figure 3). *Post hoc* analysis indicated that only 10 mg/kg metrifonate reversed the scopolamine-induced memory deficit.

Table 3 Effects of Ro4368554 and Metrifonate Treatment on Exploration Times in the ORT: (a) Time-Dependent Forgetting, (b) Scopolamine, and (c) Acute TRP Depletion

Ro4368554			Metrifonate		
Dose	**e1**	**e2**	**Dose**	**e1**	**e2**
(a) Time-dependent forgetting					
0 mg/kg	13 (1.9)	12 (1.2)	0 mg/kg	25 (1.5)	27 (2.0)
1 mg/kg	18 (2.1)	13 (1.0)	3 mg/kg	25 (2.5)	27 (1.8)
3 mg/kg	11 (1.1)	14 (1.6)	10 mg/kg	23 (1.5)	32 (2.1)
10 mg/kg	8.5 (1.1)	12 (1.1)	30 mg/kg	26 (1.9)	28 (2.3)
(b) Scopolamine					
Vehicle	25 (1.6)	29 (1.3)	Vehicle	25 (1.6)	29 (1.3)
0 mg/kg	25 (1.2)	32 (1.6)	0 mg/kg	25 (1.2)	32 (1.6)
1 mg/kg	18 (1.3)‡	25 (1.4)	3 mg/kg	28 (1.3)	33 (2.0)
3 mg/kg	32 (2.0)‡	34 (2.5)	10 mg/kg	29 (1.8)	31 (2.0)
10 mg/kg	21 (1.9)	23 (2.1)	30 mg/kg	34 (2.7)‡	32 (2.1)
TRP/dose	**e1**	**e2**	**TRP/dose**	**e1**	**e2**
(c) Acute TRP depletion					
TRP+/0 mg/kg	28 (2.3)	30 (1.6)	TRP+/0 mg/kg	28 (2.3)	30 (1.6)
TRP−/0 mg/kg	29 (1.8)	29 (1.8)	TRP−/0 mg/kg	29 (1.8)	29 (1.8)
TRP−/1 mg/kg	27 (1.8)	27 (2.0)	TRP−/1 mg/kg	20 (1.4)	27 (2.6)
TRP−/3 mg/kg	21 (1.4)	29 (2.3)	TRP−/3 mg/kg	22 (1.7)	32 (3.5)
TRP−/10 mg/kg	24 (1.7)	29 (2.2)	TRP−/10 mg/kg	20 (1.7)	25 (2.1)

Rats received injections of Ro4368554 at doses of 1, 3 and 10 mg/kg (i.p 1 ml/kg) and metrifonate at doses of 1, 3, 10 and 30 mg/kg (p.o 1 ml/kg), 60 and 30 min before T1, respectively. Mean values (±SEM) of total exploration time (s) during the first (e1) and the second trial (e2) are presented: (a) after a 24-h delay, (b) treatment of scopolamine, and (c) treatment of a TRP-free, protein-carbohydrate mixture. Differences from control group in each condition: $^{\ddagger}p<0.05$.

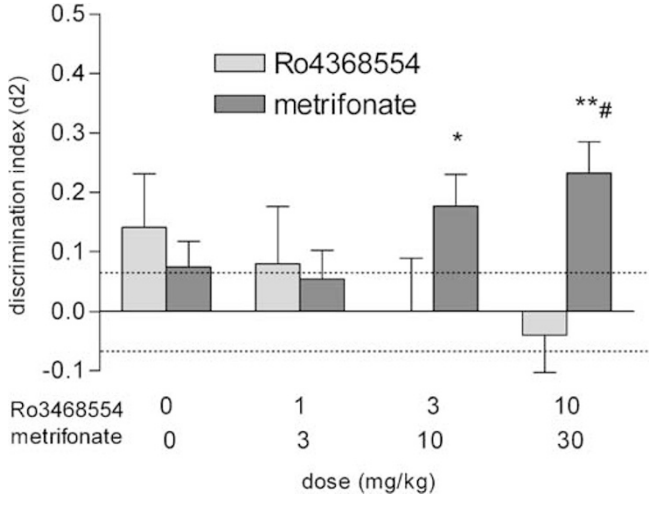

Figure 2 Effects of Ro4368554 and metrifonate on the discrimination performance in an ORt using a 24-h delay (mean + SEM). In the vehicle session, rats were treated with saline. In the drug session, rats were treated with doses of 1, ,3 and 10 mg/kg of Ro4368554 or with doses of 3, 10, and 30 mg/kg of metrifonate. Differences from virtual control group (indicated by the dotted line): $^{*}p<0.05$; $^{**}p<0.01$. Differences from vehicle group: $^{\#}p<0.05$.

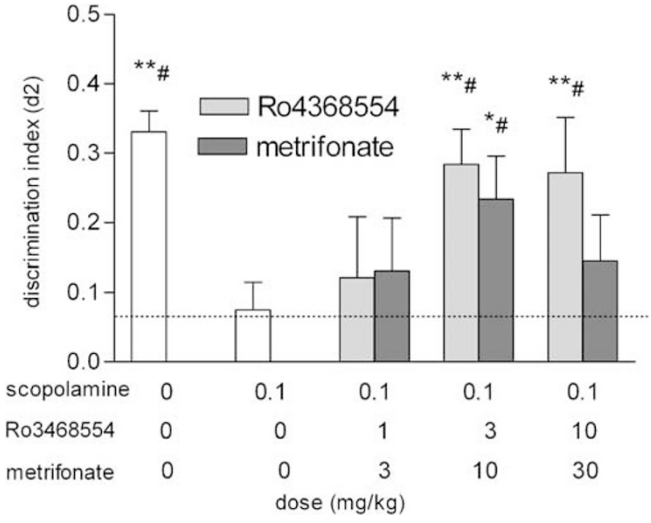

Figure 3 Effects of Ro4368554 and metrifonate after treatment with scopolamine hydrobromide on the discrimination performance in an ORT in adult male rats (mean + SEM). A 1-h interval was used between trials. In the vehicle session, rats were treated with saline. In the drug session, rats were treated with doses of 1, 3, and 10 mg/kg of Ro4368554 and with doses of 3, 10, and 30 mg/kg of metrifonate. Differences from virtual control group (indicated by the dotted line): $^{*}p<0.05$; $^{**}p<0.01$. Differences from vehicle group: $^{\#}p<0.05$.

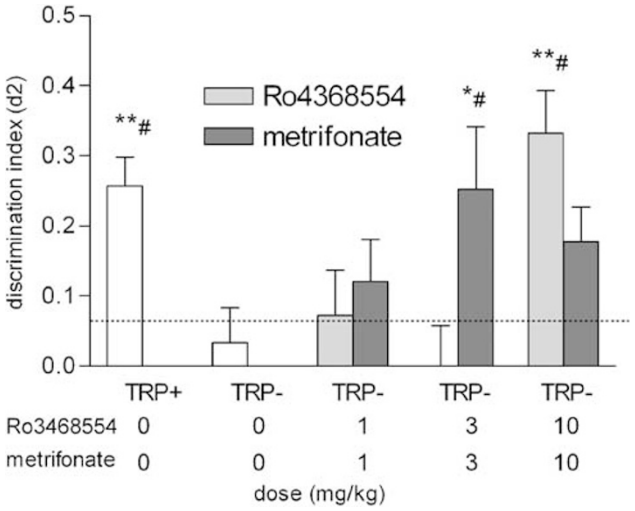

Figure 4 Effects of RO4368554 and metrifonate after TRP-free, protein–carbohydrate meal on the discrimination performance in an ORT in adult male rats (mean + SEM). A 1-h interval was used between trials. In the control session of the acute TRP depletion, rats were treated with the same meal with an addition amount of TRP (TRP+). In the vehicle session, rats were treated with saline. In the drug session, rats were treated the TRP-free, protein–carbohydrate meals and with doses of 1, 3, and 10 mg/kg of RO4368554 or metrifonate. Within session effects (different from zero virtual control group, indicated by the hatched line): $^{*}p<0.05$; $^{**}p<0.01$. Differences from TRP−/vehicle group: $^{\#}p<0.05$.

Acute TRP depletion. Metrifonate treatment affected *e*1 values in TRP-depleted rats ($F_{4,100}=3.14$, $p<0.05$; see Table 3c). Exploration levels in T2 were not affected by TRP depletion or metrifonate treatment ($F_{4,100}=0.94$, NS). An increase in explorative behavior between *e*1 and *e*2 values was found in most dose conditions. Treatment with metrifonate affected the relative discrimination index after TRP depletion ($F_{4,100}=3.79$, $p<0.01$; see Figure 4). The *d*2 value in the TRP−/vehicle-treated group was markedly reduced when compared with the TRP+/vehicle-treated group. *Post hoc* analysis revealed that the TRP depletion deficit in object recognition was significantly reversed by metrifonate at a dose of 3 mg/kg.

DISCUSSION

The aim of the present study was to investigate the cognitive-enhancing properties of the selective 5-HT$_6$ antagonist Ro4368554, and to compare its effects with those of the AChEI metrifonate in an ORT using different models of memory deficiency in adult male Wistar rats. Ro4368554 and metrifonate both reversed the memory deficit induced by scopolamine and acute TRP depletion, whereas metrifonate, but not Ro4368554, enhanced memory performance when a 24-h delay was interposed between both trials.

Impairment in Object Recognition

The discrimination performance in the ORT was dependent on the duration of the interval between T1 and T2. Consistent with results from other studies, rats accurately discriminate between the novel and the familiar object at a retention interval of 1 h, but usually not after a 24-h delay (Ennaceur and Meliani, 1992). The 24-h delay was subsequently used for time-dependent forgetting studies, whereas the 1-h delay was used for drug-induced deficiency models: scopolamine and acute TRP depletion-induced disruption of object recognition.

Treatment of scopolamine is frequently used in experimental studies to induce amnesia including novelty detection (Dodart *et al*, 1997; Ennaceur and Meliani, 1992; Pitsikas *et al*, 2001). Our data confirm the disruptive effects of scopolamine on object recognition and a dose of 0.1 mg/kg was selected for reversal studies with Ro4368554 and metrifonate. Although reversal effects of a scopolamine-induced deficit is typically interpreted in terms of an effect on cognitive function, it has been suggested that scopolamine may primarily affect sensory/attention processes rather than inducing a direct and selective effect on learning and memory processes (Blokland, 1995; Sarter *et al*, 2003). Since the present study did not address attentional or motivational functions, the possibility of attentional or sensorimotor effects cannot be completely ruled out. However, it is unlikely that nonspecific effects on motor behavior confounded the effects of scopolamine on novel object discrimination as no reduction was found in *e*1 values after scopolamine treatment, and the total exploration time in T2, which in fact gradually increased with dose. Consistent with our findings with a 0.1 mg/kg scopolamine, treatment with scopolamine at doses below 1 mg/kg s.c. have been reported to not affect motor functions (Besheer *et al*, 2001). A further remark that should be made is that many drugs, which did not have a cholinergic mechanism of action, have been shown to antagonize the effects of scopolamine. This finding suggests that a drug-induced reversal of a scopolamine deficit does not necessarily imply a cholinergic mechanism.

Recently, we found that 4 h after treating adult rats with a TRP-free, protein–carbohydrate mixture, plasma TRP levels were substantially reduced (~65%), and consequently, this resulted in decreased concentrations of 5-HT (~40%) in different brain structures (Lieben *et al*, 2004a). Moreover, it was found that plasma TRP depletion in rats caused an impaired performance in object recognition without changing affective behavior (Lieben *et al*, 2004b). This finding was in accordance with an impairment in delayed recognition several hours after a TRP-free amino acid was given to healthy humans (Riedel *et al*, 1999; Rubinsztein *et al*, 2001). These authors concluded that this effect was probably related to changes in the consolidation process when the levels of TRP were acutely reduced. In our studies, rats depleted of TRP could no longer distinguish a novel object from a familiar object they had seen 1 h before, that is, at a time interval when TRP levels were expected to be low. Based on these findings, the acute TRP depletion method was used as a 5-HTergic-deficit model for testing the effects of Ro4368554 and metrifonate on object recognition memory.

Notably, the exploration values were different in the different experiments and were sometimes affected by drug treatment. Especially, the exploration levels in the time-dependent forgetting test with Ro3438554 were relatively low. This variation in exploration levels could be related to different batches of rats, and/or scoring between experiments. Only in the scopolamine paradigm, treatment with

Ro4368554 affected exploration levels compared with the vehicle condition. To evaluate whether this change in activity interacts with performance in object discrimination, a correlation between the exploration time in the first trial (*e*1) and the discrimination index (*d*2) in the combined treatment with scopolamine and Ro4368554 was made. However, no relationship between *e*1 and *d*2 scores was found ($r = 0.014$, NS), indicating that explorative behavior in T1 does not predict memory performance. Also, in a previous study with mice, we showed that the discrimination performance based on the measure *d*2 is not dependent on the exploration level (Şık *et al*, 2003). It was suggested that exploration levels lower than 10 s may affect the reliability of the *d*2 measure. In this study, most values were clearly higher than 10 s, indicating that the discrimination measure could be measured reliably in all testing conditions. Finally, our results are comparable to other studies, showing no clear evidence that 5-HT$_6$ antagonists affect exploration times or induces locomotion deficits (Foley *et al*, 2004; King *et al*, 2004).

Effects of Ro4368554

Treatment with Ro4368554 did not improve object recognition in a time-dependent forgetting protocol, but reversed the impairment in object discrimination induced by treatment with scopolamine and the memory deficit induced by TRP depletion. These data further support the notion that 5-HT$_6$ receptor antagonists have cognition-enhancing properties (Rogers and Hagan, 2001; Woolley *et al*, 2001, 2004). Comparing the dose-related effects of Ro4368554 across both drug-induced deficit models revealed a similar pattern. At the highest dose, Ro4368554 enhanced memory performance in both scopolamine-treated and TRP-depleted rats. However, the medium dose was only effective in reversing the scopolamine-induced effect, indicating a wider active dose range in the cholinergic-deficit model. It therefore appears that the dose–response curve has shifted somewhat towards the right in the TRP depletion model as compared to the scopolamine model, suggesting that the cholinergic-deficit model is more sensitive to the effects of Ro4368554. However, higher doses of Ro4368554 need to be tested to confirm this conclusion.

The present data are consistent with those reported with Ro4368554 in a social recognition task. That is, Ro4368554 did not improve a time-dependent retention deficit but ameliorated a scopolamine-induced impairment of social memory (Szczepanski *et al*, 2002). On the other hand, a recent study showed that two 5-HT$_6$ antagonists reversed a time-dependent forgetting when injected 20 min before or directly after T1 in a novel object discrimination task (King *et al*, 2004). It is likely that differences in rat strain (Wistar *vs* Lister hooded) and delay interval (24 *vs* 4 h) may account for these differences. These findings suggest that the effects of 5-HT$_6$ antagonists on delay-dependent forgetting are less robust than the effects in a scopolamine-deficit model.

Effects of Metrifonate

In contrast to Ro4368554, metrifonate reversed retention deficits induced by a 24-h delay at the highest dose of 30 mg/kg. Furthermore, the scopolamine-induced deficit on object recognition was enhanced at a medium dose of 10 mg/kg.

Our data are consistent with the metrifonate-induced amelioration of memory performance in a wide variety of behavioral tests at doses of 10 and 30 mg/kg (Blokland *et al*, 1995; Dachir *et al*, 1997; Prickaerts *et al*, 1999; Riekkinen *et al*, 1996; Scali *et al*, 1997; van der Staay *et al*, 1996a). Furthermore, at a dose of 3 mg/kg, metrifonate reversed the retention deficit after TRP depletion to a level comparable with the TRP+ group. Since metrifonate reversed the memory deficit induced by acute TRP depletion at a lower dose than the reversal of a scopolamine-induced memory deficit, the effects of metrifonate seem to be somewhat more potent when tested in a 5-HT-mediated deficiency model. The effects of metrifonate at low doses may involve another mechanism since the minimal effective dose for reversal of the TRP depletion deficit in object recognition (3 mg/kg, p.o.) is around 10-fold lower than the reported minimal effective dose for inhibition of AChE activity (van der Staay *et al*, 1996b). Indeed, it was suggested that the cognition-enhancing effects of metrifonate may involve noncholinergic mechanism (van der Staay *et al*, 1996b), for example, inhibition of acylpeptide hydroxylase (Richards *et al*, 1999, 2000).

Neurochemical Mechanisms Involved in the Effects of 5-HT$_6$ Antagonists on Cognition

There is only a limited number of studies in which the neurochemical effects of 5-HT$_6$ antagonism have been investigated. The present data suggest that it may involve an increase in cholinergic transmission (Riemer *et al*, 2003; Shirazi-Southall *et al*, 2002). Previous behavioral studies indicated such an enhanced cholinergic neurotransmission after administration with a 5-HT$_6$ antagonist (Bentley *et al*, 1999). The present finding that Ro4368554 reversed a scopolamine-induced deficit in novel object recognition corroborates other studies showing scopolamine reversal in cognition tasks (Foley *et al*, 2004; Meneses, 2001; Woolley *et al*, 2003). These data strongly support the notion that the cognition-enhancing effects of 5-HT$_6$ antagonists involve a cholinergic mechanism.

Although the effects seem to involve a cholinergic mechanism, microdialysis studies have shown that the 5-HT$_6$ antagonist SB-271046 increases levels of glutamate and aspartate in the frontal cortex and hippocampus (Dawson *et al*, 2001). It was suggested that this effect may be mediated via an indirect effect of blockade of 5-HT$_6$ receptors on GABAergic interneurons (see also Woolley *et al*, 2004). Given the critical role of the glutamatergic system in long-term potentiation (Bliss and Collingridge, 1993), this effect on excitatory amino acids may be involved in the improved memory performance by 5-HT$_6$ antagonists, including Ro4368554. This notion is supported by a recent finding showing that glutamate is involved in the enhanced object memory performance by the 5-HT$_6$ antagonist Ro046790 (King *et al*, 2004).

Our studies in TRP-depleted animals suggest that a 5-HTergic mechanism may also contribute to the cognitive-enhancing effects of Ro4368554. However, microdialysis experiments showed that 5-HT$_6$ antagonism did not change

5-HT levels in various brain areas (Dawson *et al*, 2001; Lacroix *et al*, 2004). At least two possible alternative explanations can be offered to explain the effects of Ro4368554 in the TRP model. First, acute TRP depletion decreased levels of the amino acid citruline without affecting arginine levels (Lieben *et al*, 2004a). It has been suggested that this effect may reflect a decrease in the nitric oxide synthase activity, and concomitantly in reduced nitric oxide levels. Since glutamate and nitric oxide are closely linked in pathways associated with long-term potentiation (Bliss and Collingridge, 1993; Lynch, 2004). Since long-term potentiation is assumed to represent a physiological model for learning and memory (Lynch, 2004), this might be a potential mechanism underlying cognitive deficits in a TRP deficiency model. As mentioned earlier, 5-HT$_6$ antagonists seem to increase excitatory neurotransmission (Dawson *et al*, 2001). Consequently, if the effects of TRP depletion were mediated via a nitric oxide mechanism, the effects of Ro4368554 in the TRP depletion model might be mediated via the nitric oxide–glutamatergic pathway.

A second explanation involves an indirect effect on 5-HT. A recent study showed that the 5-HT$_6$ antagonist SB-271046 augmented the effects of amphetamine on 5-HT (and dopamine) release (Dawson *et al*, 2003). These data suggested that 5-HT$_6$ antagonism may have a modulatory —rather than a direct—effect on 5-HT neurotransmission. In the TRP model, the 5-HT system is compromised and Ro4368554 could modulate these effects. Thus, a relative increase in 5-HT neurotransmission could be established by Ro4368554 after acute TRP depletion. Preliminary data (Dr D.Bonhaus) indeed support this speculative explanation and showed that Ro4368554 increased hippocampal 5-HT levels in TRP-depleted rats. To fully understand the mechanisms by which 5-HT$_6$ antagonists exert their effects on cognition, more studies are needed to scrutinize the effects of these drugs on the modulation of neurotransmitter release.

5-HT/ACh and Memory

The 5-HTergic system is implicated in learning and memory processes, although its precise function is still a matter of debate. *In vitro* (Gillet *et al*, 1985; Maura *et al*, 1989) and *in vivo* (Leonard and Llinas, 1994; Robinson, 1983) studies suggest that a general increase in serotonergic activity leads to an inhibition in cholinergic activity. Accordingly, it would be expected that TRP depletion would result in an increase in cholinergic activity and a subsequent improvement in object recognition (cf. Prickaerts *et al*, in press). It should be noted that the changes in serotonin levels may have a dissociable effect on cholinergic activity because different 5-HT receptor subtypes, having a dissociable effect on neurotransmitters, are activated. Since TRP depletion induced a memory deficit, the effects of TRP depletion on baseline performance in the novel object recognition might not be mediated via a cholinergic component. Our behavioral data suggest that 5-HT and ACh are not inversely related in object memory, and that both neurotransmitters may affect independent processes in object memory (cf. Steckler and Sahgal, 1995). This may also apply for the effects of Ro4368554. This drug seems to reverse the cholinergic-induced memory deficit via a cholinergic mechanism and/or glutamatergic mechanism. Conversely, the reversal of 5-HTergic memory deficit by Ro4368554 might be mediated via a 5-HTergic mechanism and/or glutamatergic mechanism.

It should be mentioned that the mechanism of action of drugs should preferably be tested in a design that allows the evaluation of interaction effects (eg evaluating the effects of a drug in different testing conditions). However, the performance of the Wistar rats in the object recognition as measured by the relative discrimination index *d*2 does not exceed 0.35. On the other hand, reliable discrimination performance is usually observed at *d*2 values of about 0.20. As a consequence, the room for evaluating interaction effects is relatively small. Although the ORT is a suitable task to evaluate drug effects in different memory-deficit models, the test may be not be sensitive enough to statistically evaluate interaction effects.

In conclusion, both the serotonergic and cholinergic system may independently affect object memory in different manners. The TRP depletion method can be used to test the effects of other potential cognitive enhancers in order to increase our understanding of the role of 5-HT, and its interactions with other neurotransmitter systems, in processes of learning and memory. The present data provide further support for the notion that 5-HT$_6$ antagonists may mediate memory performance via different neurochemical mechanisms.

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