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Effects of RGH-237 (N-{4-[4-(3-aminocarbonyl-phenyl)-piperazin-1-yl]-butyl}-4-bromo-benzamide), an orally active, selective dopamine D_3 receptor partial agonist in animal models of cocaine abuse

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List of abbrevations:

AUC – area under curve

CHO – chinese hamster ovary

DTT – dithiotreithol

EDTA – ethylene diamine tetra-acetic acid

FR1 – fixed ratio one

PBS – phosphate-buffered saline

SA – self-administration

Sf9 - Spodoptera frugiperda (pupal ovary)

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ABSTRACT

RGH-237 (N-{4-[4-(3-aminocarbonyl-phenyl)-piperazin-1-yl]-butyl}-4-bromo-benzamide) is a novel potent and selective dopamine D_3 receptor partial agonist compound. It showed nanomolar affinity to both human (K_i=6.7 nmol/l) and rat (K_i=1.6 nmol/l) D_3 receptors with an intrinsic activity of appr. 50 %. It possessed several hundred-fold selectivity over the D_2 receptor. The compound bound with moderate affinity to human and rat 5-HT_{1A} receptors (K_i=136 and 245 nmol/l, respectively) and to non-selectively labelled populations of rat opiate receptors (K_i=129 nmol/l). RGH-237 proved to be practically inactive on more than 40 other targets including monoaminergic, cholinergic, GABAergic and glutamatergic receptors. In rats orally administered RGH-237 was well and rapidly absorbed yielding 41 % oral bioavailability. At its pharmacologically active dose (10 mg/kg p.o.) the brain concentration of RGH-237 reached 110 ng/g. Its blood and brain levels were sustained for 3 hours. RGH-237, at the oral dose of 10 mg/kg but not 30 mg/kg moderately though significantly inhibited the acquisition of cocaine-induced place-preference, while itself had no place-conditioning effect. The compound did not affect FR1 cocaine self-administration. In a reinstatement paradigm of cocaine self-administration the compound potently and dose-dependently blocked the cue-induced cocaine-seeking behaviour of rats at 10 and 30 mg/kg oral doses. RGH-237 did not affect seeking activity for natural rewards such as sucrose and water. The molecule did not exert notable effect on spontaneous motor activity of rats. The above properties render RGH-237 a useful pharmacological tool for investigating the therapeutic potential of dopamine D_3 partial agonism in animal models of cocaine addiction.

INTRODUCTION

In recent years a growing body of evidence has accumulated from animal studies demonstrating the importance of dopamine D_3 receptors in mechanisms of cocaine addiction. Non-selective D_2/D_3 dopaminergic agonists have been shown to mimic the effects of cocaine in drug-discrimination (Spealman, 1996) as well as in cocaine self-administration paradigms (Caine et al., 1997, Parsons et al., 1996), and their potency correlated highly with their in vitro affinity and functional activity on D_3 but not D_2 receptors (Spealman, 1996; Caine et al., 1997). In addition, nafadotride, a modestly selective D_3 receptor antagonist (Sautel et al., 1995), decreased the reinforcing effect of cocaine in the latter method (Caine et al., 1997). Furthermore, the partial D3 agonist compound, BP-897 (Pilla *et al*, 1999) and the highly D_3 selective full antagonist SB-277011-A (Reavill et al., 2000) were reported to decrease cue-induced drug-seeking behavior under a second order schedule of cocaine self-administration (Pilla *et al*, 1999; DiCiano *et al*, 2003).

More important and clinically more relevant are the findings obtained with dopamine D_3 receptor ligands in various paradigms of reinstatment of cocaine-seeking which model the human relapse phenomenon – the core problem of cocaine addiction (Katz and Higgins, 2003). In a discriminative cue-induced reinstatement model nafadotride (Weiss et al., 2001) as well as BP-897 (Cervo *et al*, 2003) suppressed cocaine-seeking. BP-897 was also shown to attenuate reinstatement of cocaine-seeking induced by contextual and stimulus related cues (Gál and Gyertyán, 2006). In addition, a non-selective D_3 partial agonist compound, a structural analogue of BP-897, was also reported to reduce the number of active lever presses induced by reintroduction of cocaine associated stimuli after extinction (Ciampiani *et al*,

2003). SB-277011-A was found to be effective in cocaine-triggered (Vorel *et al*, 2002), stress-induced (Xi et al, 2004) and cue-elicited (Gál and Gyertyán, 2006) reinstatement of cocaine-seeking paradigms.

However, D_2 preferring antagonists like haloperidol (Gál and Gyertyán, 2006) and raclopride (Crombag et al, 2002; Cervo et al, 2003) also proved to be effective in reducing cocaine-seeking behavior in cue-induced reinstatement paradigms in rats while the most D_2 selective antagonists nemonapride and eticlopride attenuated the cocaine-priming induced drug-seeking in monkeys (Khroyan et al., 2000). Therefore, from an efficacy point of view functional antagonism of both the dopamine D_3 and D_2 receptors may be potentially usable mechanism for medications aiming to prevent relapse to cocaine-seeking. However, well-known central effects are coupled to D_2 receptor antagonism which may prove to be undesirable along the treatment of cocaine addicts. On the other hand, the latest highly D_3 selective compounds showed cleaner activity profile free from marked actions on behavior (Millan et al, 2000; Reavill et al, 2000). Thus, drugs targeting the dopamine D_3 receptor may provide a more favorable clinical approach for the treatment of relapse to cocaine-use.

It was also suggested on theoretical grounds that partial agonists may be even more favourable than full antagonists in preventing relapse to drug-taking (Pulvirenti and Koob, 1994; Pilla et al., 1999; Childress and O'Brien, 2000). It is expected that a partial agonist compound acts with moderate agonism in states where natural transmitter tone is lacking or diminished, such as in the case of cocaine withdrawal, while it behaves as antagonist under conditions of increased dopamine release, as may occur during cocaine intake or in response to cocaine associated cues. Thereby it reduces craving and/or drug seeking arising from either

state. However, this concept has not been experimentally verified yet. Up to now no superiority of BP-897 over e.g. SB 277011A has been shown in cocaine abuse models. In addition, BP-897 has a non-negligible full D_2 antagonist character (Pilla et al., 1999; Wood et al., 2000) and its partial agonist nature on the D_3 receptor has also been questioned (Wicke and Garcia-Ladona, 2001; Wood et al., 2000). Since a structural analogue of BP-897, which had similar D_3/D_2 selecitivity and proved to be antagonist on the D_2 while partial agonist on the D_3 receptor, was also active in a cocaine reinstatement model while a much more D_3 selective partial agonist analogue was not, Campiani et al. (2003) even concluded that some D2 antagonist component might significantly contribute to the reduction of cocaine craving by D3 partial agonism. However, brain penetrability of the inactive analogue was not checked in their study. Obviously, further selective dopamine D3 receptor partial agonist molecules can provide great help in investigating the relevance of the partial agonism hypothesis.

Here we report the pharmacological activity of RGH-237 (N-{4-[4-(3-aminocarbonyl-phenyl)-piperazin-1-yl]-butyl}-4-bromo-benzamide, Fig. 1), which is a highly D_3 vs. D_2 selective dopamine receptor ligand with partial agonist character on both subtypes. The compound has good oral biovailability and sufficient brain penetration and shows high efficacy in cocaine addiction models.

In order to obtain a fair evaluation on the activity of RGH-237 we chose BP-897 and SB-277011 as reference substances. Results obtained with the two latter compounds in the cocaine abuse models have already been published elsewhere (Gyertyán and Gál, 2003; Gál and Gyertyán, 2003, Gál and Gyertyán, 2006), they are re-published here for the sake of comparison.

METHODS

Animals

The experimental animals were rats (*see* strain and weight under the given method) housed in a thermostatically controlled room at 24±2 °C and at relative humidity of 50±10 % on 12-h light/dark cycle (lights off from 18.00 to 6.00 hours). The animals were kept in polycarbonate cages (Lignifer Ltd., Hungary) in groups of four except those rats which underwent surgery for cocaine self-administration; they were housed individually. The rats received unlimited access to commercial pellet rat-mouse feed (ssniff R/M+H produced by Spezialdiäten GmbH, Germany), autoclaved at 105 °C and tap water throughout all experiments, except in the pre-training periods of the self-administration procedures, when they were on 23 hour water-deprivation schedule.

Animal maintenance and research were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All the procedures carried out on animals had been approved by the local ethical committee and conformed to the rules and principles of the 86/609/EEC Directive.

Drugs

RGH-237, SB-277011A (trans-N-[4-[2-(6-cyano-1,2,3, 4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-4-quinolininecarboxamide) and BP-897 (1-(4-(2-naphthoylamino)butyl)-4-(2-methoxyphenyl)-1A-piperazine HCl) were synthesized at Gedeon Richter. Cocaine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Radioligands [^{3}H]-spiperone (spec.act. 15-16 Ci/mmol), [^{3}H]-8-OH-DPAT (spec.act. 106-170 Ci/mmol) and [^{3}H]naloxone (spec.act. 63 Ci/mmol) were obtained from Perkin Elmer Life and Analytical Sciences Inc.

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(Boston, MA, USA); [7-methoxy-^{3}H]-prazosin (spec. act. 88 Ci/mmol) and [^{35}S]-GTPγS (spec. act. 1000-1150 Ci/mmol) were purchased from Amersham Radiochemicals (Buckinghamshire, UK).

In the *in vivo* studies RGH-237, SB-277011, and BP-897 were suspended in 5 % Tween-80, cocaine was dissolved in saline.

Receptor binding

Assay conditions for the individual receptors are given in Table 1. Membrane preparation for the assays were made according to the references given in the table. Incubations were stopped by rapid filtration over glass fiber filters (Brandel cell harvester), filters were rapidly washed by ice-cold buffer and radioactivity retained on the filters was determined by liquid scintillation spectrometry.

In addition to the receptor binding assays shown in Table 1 RGH-237 was tested on additional 45 neurotransmitter receptor-, ion channel-, neurotranmitter transporter- and enzyme-binding sites at 1 μM test concentration at MDS Pharma Service (Taiwan Ltd. Pharmacology Laboratories, 158 Li-Teh Road, Peitou, Taipei, Taiwan 112, R.O.C. study No. PT# 1023692; data on file at G. Richter Ltd.)

Functional activity on dopamine D_2 and D_3 receptors

Functional activity of RGH-237, BP-897 and SB-277011 was measured on recombinant human receptors by the [^{35}S]GTPγS binding method. The A9 mouse fibroblast cell line expressing the human dopamine D_{2L} receptor was purchased from ATCC. The human dopamine D_3 receptor gene was cloned and transfected into CHO cells in-house.

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Cells were collected in PBS-EDTA, centrifuged at 2000xg, homogenized (50 mM Tris, pH=7.6, 5 mM $MgCl_2$, 1 mM EDTA) and washed with centrifugation. The pellet was resuspended in a buffer (50 mM Tris, pH=7.4, 100 mM NaCl, 7 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT), aliquoted and stored at -80°C until use.

[^{35}S]GTPγS binding assay was carried out in a buffer containing 50 mM Tris (pH=7.6), 100 mM NaCl, 7 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1 μM GDP, ligand and membrane suspension (20 μg/tube for D2 and 25 μg/tube for D3 receptors). Assay mixture (in triplicates) was preincubated for 10 min at 30°C and after addition of 50 pM [35S]GTPγS incubation was continued for an additional 60 min at 30°C. The non-specific binding was measured in the presence of 10 μM GTPγS and the basal binding was determined in the presence of buffer only. Reaction was terminated by rapid filtration (UniFilter GF/B), washed four times with 1 ml ice cold buffer and radioactivity on the filters was determined by TopCount (Packard).

For determination of IC-50 values non-linear, least squares regression analysis (Data Analysis Toolbox^{TM} , MDL Information Systems, San Leandro, CA, USA) was applied by MDS Pharma. For IC-50 determinations in binding assays and evaluation of functional activity carried out in our own laboratory non-linear regression analysis was made by Origin 6.0 (Microcal Software Inc. MA, USA). For calculation of inhbitor constants (Ki) the equation of Cheng and Prusoff (1973) was used.

In vivo pharmacokinetics in rats

Pilot pharmacokinetic investigations were performed in male Wistar rats (180 - 220 g) following single intravenous, oral and subcutaneous administration of RGH-237 or reference compounds (BP-897 and SB27011). The compounds were dosed in 0.4% acetic acid solution containing 5.5% glucose. The applied doses of RGH-237 and SB27011 were 5 mg/kg for i.v.

and 10 mg/kg for oral and s.c. treatment. BP-897 was administered at the dose of 10 mg/kg by all three routes of administration.

Plasma and brain concentrations of RGH-237 and the reference compounds were determined by an HPLC-UV method. The biological samples were extracted with Cl-butane and analysed on Supelco Discovery C18 column (150x4.6 mm, 5μ) using a gradient elution. The proportion of acetonitrile/methanol (2:1) in 0.2 M aqueous ammonium acetate increased from 40 to 70 % over 15 minutes at flow rate of 1 ml/min.

Place conditioning and effect on the acquisition of cocaine induced conditioned place preference

The method is described in detail in Gyertyán and Gál (2003). Male Sprague-Dawley rats obtained from LATI, Hungary weighing 200-220 g were used in the experiments. The place conditioning box consisted of two end compartments of different colours and floor textures (for the sake of simplicity further named as black compartment and white compartment) and a narrow middle transition zone separated by removable partitions. During the *preconditioning phase* baseline place preference of the rats was determined in three daily sessions when animals were allowed to move freely in all the three compartments of the place conditioning box for 15 min. Time spent by the rat in each compartment of the place conditioning box was measured. Data from the three preconditioning sessions were averaged for each animal and used as a measure of its baseline place preference. During the next 4 days (*conditioning phase)* alternate sessions of saline- and drug-pairing took place in the morning and in the afternoon: animals received saline or drug injections and 30 min later were confined in the black (saline treatment) or white compartment (drug treatment) for 30 min. On the last day

(*test session*) untreated rats were allowed free access to both sides of the box for 15 minutes. Time spent by the rat in each compartment was measured again.

Individual differences between test session and baseline values were calculated for the drug-paired side with positive or negative differences reflecting place-preference or place aversion, respectively. Mean±SEM of individual differences was calculated and Student's t-test for dependent samples was performed for determining statistical significance.

Cocaine-induced place-preference was carried out as described above with the modification that in the conditioning phase animals were confined to the appropriate compartment immediately after cocaine (10 mg/kg ip.) or saline administration. When effects of compounds on acqusition of cocaine-induced place preference were studied following the preconditioning phase on the basis of baseline data animals were divided in a balanced way into two groups. Both groups underwent place conditioning with cocaine; one of them received drug treatment before each cocaine injection (DRUG+COC group) while the other was given saline (COC group). Both groups were treated with saline before saline-pairings. The pre-treatment times of the compounds were the same as in the place conditioning experiments. On the last day (test phase) each rat was placed uninjected into the box and allowed free access to both sides of the box for 15 minutes.

Data from the COC group were evaluated as described above in order to verify the existence of cocaine-induced place preference. Only in the case of a statistically significant cocaine effect was further analysis carried out. Times spent on the cocaine-paired side in the post-conditioning test by the COC group and the COC+DRUG group were then compared. Group means were calculated for both groups and Student's t-test for independent samples was used for statistical evaluation.

Cocaine self-administration model

The experiments were performed as described in Gál and Gyertyán (2003). Briefly, male, Long-Evans rats (Toxicoop Ltd., Hungary) weighing between 220-250 g upon arrival were used. Training and testing took place in computer-controlled operant chambers (Coulbourn Instruments) equipped with two levers and a house-light. First, animals were trained to lever press on the “active” lever for water drops in the operant chamber. Responding on the other, "inactive lever" was registered but were without consequences. Training consisted of a daily 30 min magazine training session on FR1 schedule until all the animal had learnt the task.

After acquiring the operant behaviour, all animals were implanted with a catheter into the right jugular vein under chloralhydrate (400 mg/kg) anaesthesia. Self-administration sessions began on the fifth-seventh day after the surgery. Acquisition of cocaine self-administration (0.25 mg cocaine/infusion) was established on a FR1 schedule of reinforcement in daily sessions lasting 2 hour. When the self-administration behavior became stable (defined as no more than 15% variation in the number of self-infusions during three consecutive days), rats were challenged with the dopamine D_3 receptor ligands. Animals received the drugs 30 minutes before the self-administration session by the same route as in the place conditioning studies. Animals could be re-used for drug-testing when their performance again met the stability criterion.

The individual drug infusions on the three consecutive pre-test days were averaged and taken as pre-test baseline performance, and drug effect (number of drug infusions on the test day) was expressed as percent changes compared to the baseline. Group means were then calculated from these individual percent data. For statistical evaluation paired t-test was used. Group size was 5 to 7.

Cue-induced reinstatement of cocaine-seeking

The relapse model was carried out as desrcibed in Gál and Gyertyán (2006). Briefly, the method required five phases. The first three phases (1. learning the operant task (FR1) 2. surgery and recovery 3. establishing stable cocaine self-administration) were the same as in the cocaine-self administration model. 4. period of abstinence: after a minimum of 10 days cocaine self-administration the sessions were suspended for 3 weeks Animals went through the abstinence period in another room which was free from cocaine and any environmental or technical cues which could be associated with the drug-intake. 5. reinstatement: when the abstinence period expired, animals were re-introduced to a 30 min long extinction session where all the environmental and reinforcement-contingent cues were the same as in the acquisition phase except that the lever presses were not reinforced with cocaine infusions. Animals were assigned to separate treatment groups and 30 min prior to the reinstatement session received saline per os or various doses of the tested D_3 ligands.

All drug treated groups consisted of 8 rats (saline treated group: n=14) matched for their stable cocaine intake during training. Mean±SEM of number of lever pressings on the reinforced lever during the test session was calculated in each treatment group. Data were analyzed by one-way ANOVA with treatment groups as the between group factor. Whenever a significant effect was found, *post hoc* comparison was done using Duncan's multiple range test.

Cue-induced reinstatement of sucrose-seeking

The experimental procedure mirrored the previous one in every respect with the exception of surgery. Animals "self-administered" 10 % sucrose drops orally under FR1 schedule in the

operant boxes. All the environmental cues were the same as in the cocaine self-administration model except that the infusion harness and pump were absent and the reward were given through a liquid delivery system. After the stable sucrose-administration behaviour had established rats went through a three week long abstinence period from sucrose. In the reinstatement phase when drug effects were tested all the environmental cues were the same except that lever pressing was not reinforced with sucrose. Animals were assigned to separate treatment groups and 30 min prior to the reinstatement session received saline per os or various doses of the tested D_3 ligands.

All drug-treated groups consisted of 8 rats (in the pooled saline treated group: n=35) matched for their stable cocaine intake during training. Mean±SEM of number of lever pressings on the reinforced lever during the test session was calculated in each treatment group. Data were analyzed by one-way ANOVA with treatment groups as the between group factor. Whenever a significant effect was found, *post hoc* comparison was done using Duncan's multiple range test.

Cue-induced reinstatement of water-seeking

In the experiment with water reward water-deprived animals learnt to self-administer water drops under FR1 schedule during the 30 min session as it was detailed in description of pre-training phase above. After stable responding had established animals had unlimited access to water for 24 hours in their home cages. Then they were reintroduced the operant chambers (test session) and allowed to press the previously active lever: However, responding was not reinforced but the empty liquid dipper was lifted up.

Thirty min prior to this test session animals were assigned to separate treatment groups and received per os saline or various doses of the test compounds. Group size was 7-8 in the drug-

treated groups and 35 in the saline treated (pooled) control group. Mean±SEM of number of lever pressings on the reinforced lever during the test session was calculated in each treatment group. Data were analyzed by one-way ANOVA with treatment groups as the between group factor. Whenever a significant effect was found, *post hoc* comparison was done using Duncan's multiple range test.

Effect on spontaneous motor activity

Male Wistar rats weighing 160-180 g were used in the experiments. Spontaneous locomotor activity was measured in an activity monitor working with infrared photobeams which detected both horizontal and vertical (rearing) activity. Thirty minutes after the oral administration of RGH-237, SB 277011 or vehicle, animals were individually placed in the experimental cages and horizontal and vertical movements were recorded for 30 minutes.

Means ± SEM of horizontal and vertical activity counts were calculated in each treatment group. Statistical significance between group means was determined by ANOVA followed by post-hoc Duncan test. The percentage inhibition of horizontal or vertical activity were also calculated for each dose.

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RESULTS

Receptor binding profile of RGH-237

The compound RGH-237 showed nanomolar affinity to rat dopamine D_3 receptors with more than 1000-fold selectivity over the D_2 receptor (Table 2). It showed negligible activity on α_1-adrenergic receptors. SB 277011 exhibited similar profile while BP-897 – though it had stronger binding to rat D_3 receptor than the other two molecules – showed weaker selectivity over the dopamine D_2 and especially over the α_1 receptor. RGH-237 bound with moderate affinity to non-selectively labelled populations of rat opiate receptors and weakly to rat 5-HT_{1A} receptors (Table 2).

Among human targets RGH-237 showed nanomolar affinity to cloned hD_3 dopamine receptors (K_i=6.7 nmol/l) while it was found to be inactive on hD_2 receptors (IC_{50} >1000 nmol/l). Besides its D_3 receptor activity the compound possessed weak - moderate affinity to human 5-HT_{1A} receptors (K_i=136 nmol/l).

In addition, RGH-237 was examined on additional 45 neurotransmitter receptor-, ion channel-, neurotranmitter transporter- and enzyme-binding sites (MDS Pharma Service - Taiwan Ltd. Pharmacology Laboratories, 158 Li-Teh Road, Peitou, Taipei, Taiwan 112, R.O.C. study No. PT# 1023692; data on file at G. Richter Ltd.) and at concentration of 1 µM it produced less than 60 % displacement at guinea pig H1 receptors (58%), human serotonin transporter (57%), human sigma-1 receptors (50%), human serotonin 5-HT_{2A} and 5-HT_{2B} (45-45 %) receptors and less than 30 % displacement at the remaining binding sites. (The complete list of binding sites can be found in Supplemental Table 1.)

Functional activity of RGH-237 on dopamine D2 and D3 receptors

The results of a typical [^{35}S]GTPγS binding experiment with RGH-237 and the natural agonist, dopamine is demonstrated in Figure 2. In Table 3 data obtained with these two compounds, BP-897 and SB-277011 are summarised. The data indicate that RGH-237, in comparison with dopamine, behaved as a partial agonist at both the D_{2L} and the D_3 receptor. Its intrinsic activity (E_{max}) was found to be half of that of dopamine (0.54 and 0.52 for D_{2L} and D_3 receptors, respectively). RGH-237 showed 40 fold selectivity toward D_3 receptors.

As a partial agonist RGH-237 was able to inhibit the dopamine-induced [^{35}S]GTPγS binding but only up to the level of its own intrinsic activity (Fig. 3). Its IC_{50} value was found to be 28 nM (Table 3.).

In our test system BP-897 proved to be pure antagonist on both the D_2 and the D_3 receptor with 10 fold selectivity in favour of the latter (Table 3). The compound SB 277011 was found to be a highly selective (~120 fold) full D_3 receptor antagonist.

In vivo pharmacokinetics of RGH-237 in rats

Following i.v. administration of RGH-237 to rats the compound exhibited moderate plasma clearance of approximately 7.0 ml/min/kg. The volume of distribution of 0.9 l/kg did not indicate extensive tissue binding of the compound. In accordance with these parameters the plasma concentrations were very high even at the last investigated time point of 5 hours. Elimination of RGH-237 was characterised with apparent terminal half-lives ($t_{1/2}$) of approximately 1.4 and 2 hours after i.v. and p.o. administration, respectively (Table 4 and Fig. 4).

Oral and s.c. absorption of RGH-237 was relatively rapid with $t_{max} \leq 1$ hour (Table 4). The extent of absorption was high enough for both routes with oral bioavailability of 41% and s.c. bioavailability of 51%, however due to poor brain penetration the brain levels were rather low albeit sustained (Table 4 and Fig. 4).

Plasma levels of RGH-237 were much higher than those of BP 897 (about 100 fold higher after p.o. and 12 fold higher after s.c. administration), mainly because the oral absorption of RGH-237 was much better than that of BP-897. This reference compound showed very poor oral absorption. The s.c. bioavailabilities of the two compounds did not differ considerably, though (Table 4). The brain penetration of BP-897 was much higher than that of RGH-237 therefore similar plasma concentrations may represent much higher brain levels for BP-897 than for RGH-237.

SB 277011 showed similar kinetic behaviour to that of RGH-237 (Table 4).

Place conditioning and effect on the acquisition of cocaine induced conditioned place preference

Fig. 5 shows the place conditioning effects of D_3 receptor ligands. RGH-237 showed slight aversive property at the dose of 10 and 30 mg/kg. BP-897 administered ip. at 0.5 mg/kg dose had no effect on place conditioning while at the dose of 1 mg/kg it caused significant place-aversion. SB-277011 at the dose of 5 and 20 mg/kg did not produced notable effect on place conditioning.

Cocaine, at the dose of 10 mg/kg ip. induced significant place-preference in all the 6 experiments measuring the effects of dopaminergic compounds on cocaine-induced place preference (Fig. 6). The increase from baseline in the time spent in the cocaine-paired compartment (effect size) ranged from 73 to 215 sec (mean 129 sec).

The lower dose of RGH-237 significantly inhibited the cocaine induced place preference. None of the doses of BP-897 could inhibit the place preference induced by cocaine, moreover the low dose slightly enhanced the effect of cocaine. SB-277011 was ineffective in influencing the place preference elicited by cocaine (Fig. 6).

Cocaine self-administration model

RGH-237 at the dose of 10 and 30 mg/kg and SB-277011 at doses of 5 and 20 mg/kg did not influence the cocaine self-administration under FR1 schedule (data not shown). BP-897 at the dose of 1 mg/kg slightly (26 %), but significantly enhanced the lever pressing for cocaine while the 0.5 mg/kg dose was ineffective.

Cue-induced reinstatement of cocaine-seeking

Even after three weeks of abstinence when saline treated animals met the secondary cues associated with cocaine infusions a strong drug-seeking behaviour appeared: the rats continuously pressed the previously reinforced lever, despite the fact that lever presses were not reinforced (Fig. 7).

ANOVA revealed significant treatment effect on the number of lever presses in the test session ($F(6,55)=5.74$, $p= 0.00011$). *Post-hoc* comparisons showed that administration of the 10 mg/kg and the 30 mg/kg doses of RGH-237 significantly and dose dependently inhibited the secondary cues-induced cocaine-seeking behaviour comparing to the control group. BP-897 at the dose of 1 mg/kg also significantly attenuated the number of lever presses while there was no significant effect of the lower dose of this compound. Both doses of SB-277011-A produced significant inhibition in the drug seeking behavior although without dose-dependence (Fig. 7).

Cue-induced reinstatement of sucrose-seeking

After three weeks of abstinence secondary cues paired with previous sucrose-taking were also able to elicit reward-seeking behaviour in the reinstatement session although to a lesser extent than cocaine could (44.9±4.1 responses/session, Fig. 8).

However, in contrast with their effects in the cocaine reinstatement model, neither RG-237 nor BP-897 and SB-277011 significantly influenced this behaviour ($F(5,68)=1.10$, $p=0.422$) (Fig. 8).

Cue-induced reinstatement of water-seeking

In spite of the fact that the rats were not water-deprived during the test session, animals were actively engaged in pressing the previously reinforced lever (47.4±5.0 presses/session) (Fig. 9).

Similarly to their effects on sucrose-seeking none of the tested compounds could significantly influence the lever presses induced by secondary cues associated with previous water self-administration ($F(5,68)=0.840$, $p=0.526$). (Fig. 9). Only the 1 mg/kg dose of BP-897 caused a moderate (44%) though non significant inhibition.

Effect on spontaneous motor activity

RGH-237 did not affect the locomotor activity of rats up to the dose of 30 mg/kg (Table 5, $F(3,56)=0.817$). At 10 mg/kg p.o. it produced a moderate, non-significant reduction in vertical movements ($F(3,56)=0.158$).

SB 277011 did not affect any component of motor activity of rats in the dose range of 13.5-30 mg/kg p.o. ($F(3,36)=0.955$ for horizontal and $F(3,36)=0.375$ for vertical activity).

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DISCUSSION

On the basis of the receptor binding results RGH-237 has high affinity and selectivity for either human or rat D_3 receptors. In rat the compound showed excellent (>1000-fold) selectivity over the D_2 and adrenergic α_1 receptors, and still considerable selectivity over opiatergic (80-fold) and 5-HT_{1A} serotonergic (150-fold) receptors. The human receptor profile of RGH-237 was similar to that in the rat though with proprotionally less selectivity ratios (D_3/D_2: > 150, D_3/5-HT_{1A} = 20). No appreciable affinity (IC-50 > 1000 nM) was noted at 45 other receptors, ion-channels and enzymes.

With respect to selectivity over dopaminergic and adrenergic receptors SB 277011 resembled RGH-237 whereas BP-897 had lower selectivity to D_2 and especially to α_1 receptors (165-fold and 50 fold, respectively). These data are in good accordance with the literature. SB 277011 was found to be highly selective over any other catecholaminergic receptor (Reavill et al., 2000) while the D_3/D_2 selectivity of BP-897 spans from 38-fold (Wood et al., 2000) through 66-fold (Pilla et al., 1999) to 131-fold (Cussac et al., 2000). BP-897 was shown to have similar (Pilla et al., 1999) or even lower (Cussac et al., 2000) selectivity to α_1 and α_2 receptors. Regarding the 5-HT_{1A} affinity and selectivity RGH-237 lies between SB 277011, which is inactive on this receptor (K_i > 5000 nmol/l, Reavill et al., 2000), and BP-897, which was found to have moderate (K_i=84 nmol/l, Pilla et al., 1999) to marked (K_i=4.8 nmol/l, Cussac et al., 1999) affinity to the 5-HT_{1A} receptor. In contrast to RGH-237, neither SB 27701 nor BP-897 has measurable affinity to opiate receptors (Pilla et al., 1999; Reavill et al., 2000).

In functional *in vitro* assays RGH-237 proved to be partial agonist both on human D_3 and D_2 dopamine receptors with about 50 % intrinsic activity. Unlike RGH-237, both SB 277011 and BP-897 were found to be full antagonist on both receptor subtypes in our experiments.

Our finding with BP-897 on the D_3 receptors contrasts the results of the Sokoloff group which described it as a partial D_3 receptor agonist with 53% intrinsic activity (Pilla et al., 1999). A possible explanation for the discrepancy may be the use of a different endpoint (thymidine incorporation *vs.* GTPγS binding in our lab). However, three other studies also showed the compound to be a full D_3 receptor antagonist measured either by extracellular acidification rate (Wood et al., 2000) or by GTPγS binding (Cussac et al., 2000; Wicke and Garcia-Ladona, 2000). The latter authors even demonstrated the antagonist charcater of BP-897 *in vivo*, namely on quinpirole-induced inhibition of firing rate of dopaminergic neurons in the substantia nigra (Wicke and Garcia-Ladona, 2000). On the other hand, BP-897 was unequivocally shown to be a full antagonist on the D_2 receptor (Pilla et al., 1999; Cussac et al., 2000; Wood et al., 2000) similarly to our own results. Regarding the full D_3 antagonist nature of SB 277011 our results are in good accordance with the published findings of Reavill et al. (2000) who used acidification rate as endpoint.

Taking all the above data into account we consider RGH-237 as a selective D_3 partial agonist while SB 277011 as a selective D_3 full antagonist compound. BP-897 probably behaves as a D_3 preferring D_3/D_2 dual antagonist in native conditions, though its partial agonist-like action cannot be excluded on certain cell-types and/or in certain physiological states.

In rats RGH-237 was absorbed well and rapidly not only after parenteral but also after oral administration. Its blood-level was sustained up to 3 hours. In contrast, BP-897 practically proved to be an orally non-absorbable compound. However, following subcutaneous injection

the two compounds yielded similar bioavailability values. SB 277011 showed excellent oral absorption in accordance with the results of Reavill et al. (2000).

In contrast to its good intestinal penetration cerebral penetration of RGH-237 was rather poor. The resulting low brain concentration explains the relatively high effective doses of RGH-237 in the cocaine models despite its low K_i value on the D_3 receptor.

BP-897 showed much better brain penetration than RGH-237 yielding a brain/plasma concentration ratio of five. The high brain level on the one hand justifies the relatively low effective dose of BP-897 in the cocaine models while on the other hand it raises the possibility that the observed effects of the compound may have involved the dopamine D_2 receptor as well. From the concentration measured at 10 mg/kg sc. dose a brain concentration of 510 nM can roughly be estimated at the 1 mg/kg sc. dose of the compound. This is more than 10-fold higher than its K_i value (33 nM) on the D_2 receptor. Even if we take into consideration the practical and theoretical limits of such calculations it seems likely that not only D_3 but also D_2 receptor mediated actions may underly the observed *in vivo* effects of BP-897.

In contrast, in the case of RGH-237 the pharmacokinetic measurements suggest that the compound penetrates into the brain at concentrations providing sufficient occupancy of the D_3 receptors while maintaining selectivity over D_2 receptors. Therefore RGH-237 is especially suitable for testing the effect of D_3 partial agonism in various models of cocaine use.

RGH-237, like SB 277011, did not produce significant place conditioning effects while BP-897, at its higher dose induced moderate but significant place aversion. Since the receptor profile of BP-897 is not very clean it is difficult to tell what mechanism(s) may lie behind its effect. Since D_3 agonist compounds such as PD-128907 and 7-OH-DPAT were shown to

cause significant place aversion (Gyertyán and Gál, 2003) a possibility may be that in this peculiar paradigm BP-897 behaves as a D_3 partial agonist. However, RGH-237 which is undoubtedly a partial agonist showed only a slight tendency to induce place aversion. Another receptor candidate is the adrenergic α_1 receptor to which BP-897 showed affinity comparable to that of D_3 with pure antagonist functional activity (Cussac et al., 2000). However, the selective α_1 antagonist prazosin was shown to be inactive in the place conditioning paradigm (Tzschenke, 1998; Sahraei et al, 2004). BP-897 has still considerable affinity to 5-HT_{1A} and α_2 receptors being partial agonist on the former and antagonist on the lattter (Cussac et al., 2000). However, 5-HT_{1A} agonists and partial agonists caused place preference (Tzschenke et al., 1998) while there are conflicting data with α_2 antagonists: idazoxan induced place preference while yohimbine was shown to produce place aversion or no effect (Tzschenke et al., 1998; Morales et al., 2001; Sahraei et al, 2004). Finally, even the D_2 antagonist character of BP-897 does not give a clue because the highly potent D_2 antagonist haloperidol reproducibly showed inefficacy in place conditioning (Tzschenke, 1998).

In the cocaine-induced place preference model where the compounds were administered in the acquisition phase of conditioning both doses of RGH-237 produced small inhibition which proved to be statistically significant at the lower dose. SB-277011 and BP-897 did not induce significant changes in cocaine-induced place preference, although the lower dose of BP-897 nearly significantly increased the action of cocaine. However, its effect size, just as that of RGH-237, was quite small. Because of the low magnitude and the lack of dose-dependence we do not assign biological significance to either of these effects. According both to the literature (for review *see* Tschzenke, 1998) and to our other results (Gyertyán and Gál, 2003) neither the various D_3 antagonists nor D_3 agonists could notably influence the cocaine-induced place preference. It appears that RGH-237 does not differ from them.

RGH-237, like SB-277011, did not influence the FR1 cocaine self-administration behaviour. In contrast, the 1 mg/kg dose of BP-897 slightly but significantly increased the number of infusions, which means that it inhibited the rewarding effect of cocaine. While for SB-277011 our results are in accordance with findings published in the literature (DiCiano et al., 2003) it is not the case with BP-897. Pilla et al. (1999) reported BP-897 to be ineffective on cocaine self-administration under continuous reinforcement at the dose of 1 mg/kg ip. and below. Since the effect of BP-897 was not very robust in our study this discrepancy may well be due to some methodical differences between the two laboratories. Given that both the full antagonist SB-277011 and the partial agonist RGH-237 were ineffective in the same paradigm it seems unlikely that the effect of BP-897 on cocaine self-administration was mediated via the D_3 receptor. On the other hand since haloperidol and other D_2 antagonists do increase cocaine SA under fixed ratio schedule (Caine et al., 2002; Corrigall and Coen, 1991; Gál and Gyertyán, 2003) an action of this nature may provide a more plausible explanation for the observed effect of BP-897. However, since this compound was shown to have similar or higher affinity to α_1, α_2 and 5-HT_{1A} receptors than to the D_2 receptor these actions also cannot be excluded.

Findings in the cocaine place preference and cocaine self-administration models suggest that RGH-237, like SB-277011, does not exert a direct blocking action on the interoceptive cue/rewarding property of cocaine.

RGH-237 produced dose-dependent and robust inhibition in the secondary cue-induced reinstatement of cocaine self-administration. 30 mg/kg of the compound decreased the drug-seeking behaviour to 17 % of the control. BP-897 also dose-dependently reduced cocaine-

seeking however, with weaker efficacy. SB-277011 proved to be active as well, exerting 70% inhibition at its lower dose.

Our results with SB-277011 add up to the growing list of findings with this molecule demonstrating the efficacy of selective D_3 antagonism in various forms of cocaine-seeking reinstatement models: cocaine-triggered (Vorel et al., 2002), cue-induced (Cervo et al., 2006) and stress-induced (Xi et al., 2004) reinstatement. But what about the idea of D_3 partial agonism? Although BP-897 was also shown to prevent cue-induced reinstatement of cocaine-seeking (Cervo et al., 2003, Gál and Gertyán, 2006) results obtained with this compound do not provide convincing evidence for the utility of D_3 partial agonism because – as we pointed out above – both its partial agonist nature and its in vivo D_3 selectivity are questionable. The D_2 antagonist as well as the α_1 antagonist character of BP-897 may also have played role in its reinstatement inhibitory action since both types of antagonists were shown to decrease reinstatement of cocaine-seeking (Gál and Gyertyán, 2006; Zhang and Kosten, 2005). Moreover, as a much more D_3 selective structural analogue of BP-897, which proved to act as partial agonist (measured by GTPγS binding), was not able to inhibit cue-induced reinstatement of cocaine-seeking (however, brain levels of the compound were not checked) it has been questioned whether D_3 partial agonism alone could be an effective mechanism for preventing reinstatement of cocaine-seeking (Campiani et al., 2003). Within this context it is of great importance that RGH-237, a selective D_3 partial agonist was shown to be highly effective in this model thereby providing supporting evidence for the applicability of partial D_3 agonism in preventing reinstatement of cocaine-seeking.

In order to check the cocaine-specificity of the reinstatement inhibitory actions of RGH-237 we studied the effect of the compound on secondary cue-induced natural reward-seeking

behaviour. For reinforcement we used sucrose as natural reward with hedonic property and water as natural reward with homeostatic property. RGH-237, similarly to SB-277011 and BP-897, did not influence either type of reward seeking behavior. These results suggest that in the reward seeking models the effect of RGH-237, like those of the other two D_3 compounds, is cocaine-specific.

Our results demonstrate that not only dopamine D_3 full antagonists but selective D_3 partial agonists could be effective in preventing the cue induced relapse in the clinic and may have therapeutic potential for the treatment of cocaine abuse.

RGH-237, like SB-277011, did not exert notable effect on spontaneous motor activity in rats. (BP-897 was not tested in this method.) Selective D_3 antagonists including SB-277011 were shown to have negligible effect on motor activity in rats (Reavill et al., 2000; Millan et al., 2000; Gyertyán and Sághy, 2004). According to our results with RGH-237 this observation can be extended to D_3 partial agonists, too. Furthermore, these results further confirm that the decrease in response rate caused by the compounds in the cocaine reinstatement paradigm did not result from a non-specific sedative action.

In conclusion, RGH-237 is a real partial agonist on dopamine D_3 receptors with an intrinsic activity of about 50 % and with greater than 1000 fold selectivity over the D_2 receptor. Orally administered the compound penetrates the brain at sufficient concentration. Accordingly, it proved to be highly effective in a cue-induced reinstatement of cocaine-seeking model. RGH-237 may be considered as a useful pharmacological tool for studying the consequences of partially stimulating the dopamine D_3 receptor.

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FOOTNOTES:

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LEGENDS FOR FIGURES

Fig. 1. Chemical structure of RGH-237 (M_{wt}: 459.39)

Fig. 2. Concentration-response curves of dopamine (●) and RGH-237 (□) on stimulation of recombinant human dopamine D_3 (A) and D_{2L} receptors (B).

Fig. 3. Concentration-response curves of RGH-237 alone (□) and in the presence of 1 μM dopamine (●) using recombinant human dopamine D_3 receptor.

Fig. 4. Mean plasma and brain concentrations of RGH-237 in rats after single i.v., p.o and s.c administration of the compound

Fig. 5. Place conditioning effect of RGH-237, BP-897 and SB 277011. Data are mean±SEM values of individual differences between times spent in the drug-paired compartment during the pre-conditioning phase (baseline = three days' average) and the post-conditioning test. (Mean baseline values of the six eperiments varied between 215 and 332 sec with a mean of 284 sec.) Group sizes are indicated by numbers at the bottom of each column. * $P<0.05$, statistically significant difference pre- vs. post-conditioning values (paired t-test). (*BP-897 and SB 277011 data are reprinted with permission from Gyertyán and Gál, 2003).*

Fig. 6. Effect of RGH-237, BP-897 and SB 277011 on the acquisition of cocaine-induced place preference. Data are means±SEM values. Gray columns represent the COC groups, patterned columns show the data of the DRUG+COC groups (see Methods for details). Doses and routes of administration for the various compounds administered

prior to cocaine injections are shown on the horizontal axis. Group sizes are indicated by numbers in each pair of columns. (COC and DRUG+COC groups always were of equal size.) * P<0.05, statistically significant difference COC vs. DRUG+COC group (t-test for independent samples). (*BP-897 and SB 277011 data are reprinted with permission from Gyertyán and Gál, 2003).*

Fig. 7. Effect of RGH-237, BP-897 and SB 277011 on cue-induced cocaine-seeking behaviour in a reinstatement session following 3 weeks period of abstinence. Data are mean±SEM values. *, ** and ***: p<0.05, p<0.01 and p<0.001, respectively, statistically significant difference between the drug-treated and the control groups (ANOVA followed by Duncan-test). (*BP-897 and SB 277011 data are reprinted with permission from Gál and Gyertyán, 2006).*

Fig 8. Effect of RGH-237, BP-897 and SB 277011 on sucrose-seeking behaviour in a reinstatement session following 3 weeks period of abstinence. Data are mean±SEM values. (*BP-897 and SB 277011 data are reprinted with permission from Gál and Gyertyán, 2006).*

Fig 9. Effect of RGH-237, BP-897 and SB 277011 on water-seeking behaviour of non-water-deprived rats in a test session 24 hours after the last water self-adminsitration session. Data are mean±SEM values.

Table 1. Summary of binding assays conditions

Receptor	Species	Cell/tissue (amount/assay)	Incubation buffer (mM)	Incubation time; temp.; pH	Radioligand (nM)	Non-specific (μM)	Reference
rD_3	rat	Sf9 (10 μg)	Tris-HCl 50; $MgCl_2$ 5, EDTA 5; KCl 5; $CaCl_2$ 1.5; NaCl 120;	60 min; 27° C; pH:7.4	[^{3}H]spiperone (0.4)	haloperidol (10)	RBI Biosignal Cat No. BSR-D3R
hD_3*	human	CHO (10 μg)	Tris HCl 50	120 min; 37° C; pH 7.4	[^{3}H]spiperone (0.7)	S(-)sulpiride (25)	Sokoloff et al., 1990
rD_2	rat	striatum (1.25 mg)	Tris-HCl 50; $MgCl_2$ 1, EDTA 5; KCl 5; $CaCl_2$ 2; NaCl 120;	15 min; 37° C; pH 7.4	[^{3}H]spiperone (0.5)	(±)sulpiride (10)	Seeman, 1984
hD_{2L}*	human	CHO (20 μg)	Tris-HCl 50; $MgCl_2$ 2, EDTA 5; KCl 5; $CaCl_2$ 2; NaCl 120;	120 min; 25° C; pH 7.4	[^{3}H]spiperone (2.0)	haloperidol (10)	Hayes et al., 1992
rAlpha-1	rat	cerebral cortex (32 mg)	Tris HCl 50	30 min; 23° C; pH 7.4	[^{3}H]prazosin (0.5)	phentolamine (10)	Greengrass and Bremner, 1979
r5-HT_{1A}	rat	hippocampus (0.45 mg)	Tris-HCl 50; $CaCl_2$ 4; ascorbic acid 0.1%; pargyline 0.01;	15 min; 37° C; pH 7.7	[^{3}H]8-OH-DPAT (2.0)	serotonin (10)	Hall et al., 1985
h5-HT_{1A}*	human	CHO (8 μg)	Tris-HCl 50;	60 min; 25° C; pH 7.4	[^{3}H]8-OH-DPAT (1.0)	metergoline (10)	May et al., 2003
Opiate (non-selective)*	rat	whole brain (7.5 mg)	Tris-HCl 50;	40 min; 25° C; pH 7.7	[^{3}H]naloxone (1.0)	naloxone (1)	Pasternak et al., 1975

*: assays were carried out by MDS Pharma Service (Taiwan); all other assays were done in-house.

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Table 2. Affinity of RGH-237, BP-897 and SB 277011 to rat dopamine D_3, D_2, adrenergic α_1, opiate and 5-HT_{1A} receptors

	RGH-237		**BP-897**		**SB 277011A**	
	K_i (nM)	Selectivity[a]	K_i (nM)	Selectivity[a]	K_i (nM)	Selectivity[a]
D_3	1.6	1	0.2	1	4.1	1
D_2	> 2900	> 1800	33	165	> 2900	> 700
α_1	1816	1135	10	50	3873	945
opiate	129	81				
5-HT_{1A}	245	153				

a: Ki for the receptor divided by Ki for D_3 receptor

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Table 3. Summary of data obtained with dopamine, RGH-237, BP-897 and SB-277011 in the in vitro [^{35}S]GTPγS binding assay.

Data are means±SD values. Numbers in brackets represent the number of measurements. n.d. – not determined

	hD_{2L}			**hD_3**		
	E_{MAX} (%)	**EC_{50} (nM)**	**IC_{50} (nM)**	**E_{MAX} (%)**	**EC_{50} (nM)**	**IC_{50} (nM)**
dopamine	100 (24)	426 ±222 (24)	–	100 (24)	130 ±77 (50)	–
RGH-237	54 ±8.3 (3)	346 ±191 (3)	n.d.	52 ±9.0 (3)	8.7 ±7.6 (3)	28 (1)
BP-897	6 (1)	–	94 (1)	4.6 ±1.9 (2)	–	8.9 ±11 (2)
SB-277011	n.d.	n.d.	8718 ±2474 (3)	-16 ±12 (2)	–	72 ±52 (2)

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Table 4. Pharmacokinetic parameters of RGH-237, BP-897 and SB 277011

Compound	Dose 10 mg/kg	$t_{1/2,\ plasma}$ (hour)	$C_{max,\ plasma}$ (ng/ml)	$C_{max,\ brain}$ (ng/g)	$t_{max,\ plasma}$ (hour)	Bioavail-ability %	Brain /Plasma Ratio[a]
RGH-237	10 (p.o.)	2.0 ±0.7	2876 ±371	110	0.8 ±0.4	41%	0.047
RGH-237	10 (s.c.)	1.8±0.5	4397±2041	*n.d*	1.0±0	51 %	*n.d.*
BP-897	10 (p.o.)	*n.c.*	29.4 ±10.6	*n.d.*	0.55 ±0.41	*n.c.*	*n.c.*
BP-897	10 (s.c.)	1.3	344 ±39.5	2119	0.25±0	36 %	4.9
SB 277011	10 (p.o.)	1.9 ±0.4	2166 ±197	*n.d.*	1.0 ±0	63%	*n.d.*

a: $AUC_{last,brain}/AUC_{last,plasma}$
n.d.: not determined; *n.c.*: not calculated

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Table 5. Effect of RGH-237 and SB 277011 on spontaneous motor activity of rats

Data are means±SEM of numbers of beam interruptions.

		Horizontal movements		**Vertical movements**	
Compound	*n*	*Counts*	*Effect*	*Counts*	*Effect*
RGH-237					
saline p.o.	30	1629.3 ±75.06		186.7 ±14.92	
3 mg/kg	10	1716.6 ±143.73	+5 %	192.9 ±20.71	+3 %
10 mg/kg	10	1566.7 ±178.32	-4 %	130.1 ±19.97	-30 %
30 mg/kg	10	1544.2 ±118.18	-5 %	171.6 ±14.03	-8 %
SB 277011					
saline p.o.	10	835.9 ±109.0		99.0 ±15.6	
13.5 mg/kg	10	791.7 ±70.0	-5 %	102.2 ±11.9	+3 %
20 mg/kg	10	786.6 ±50.4	-6 %	74.6 ±8.0	-25 %
30 mg/kg	10	819.6 ±30.7	-2 %	94.1 ±11.1	-5 %

Fig. 1

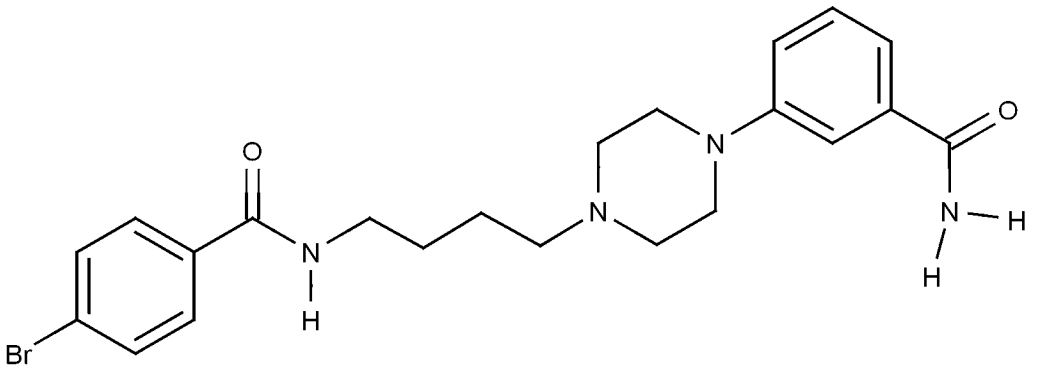


Fig. 2

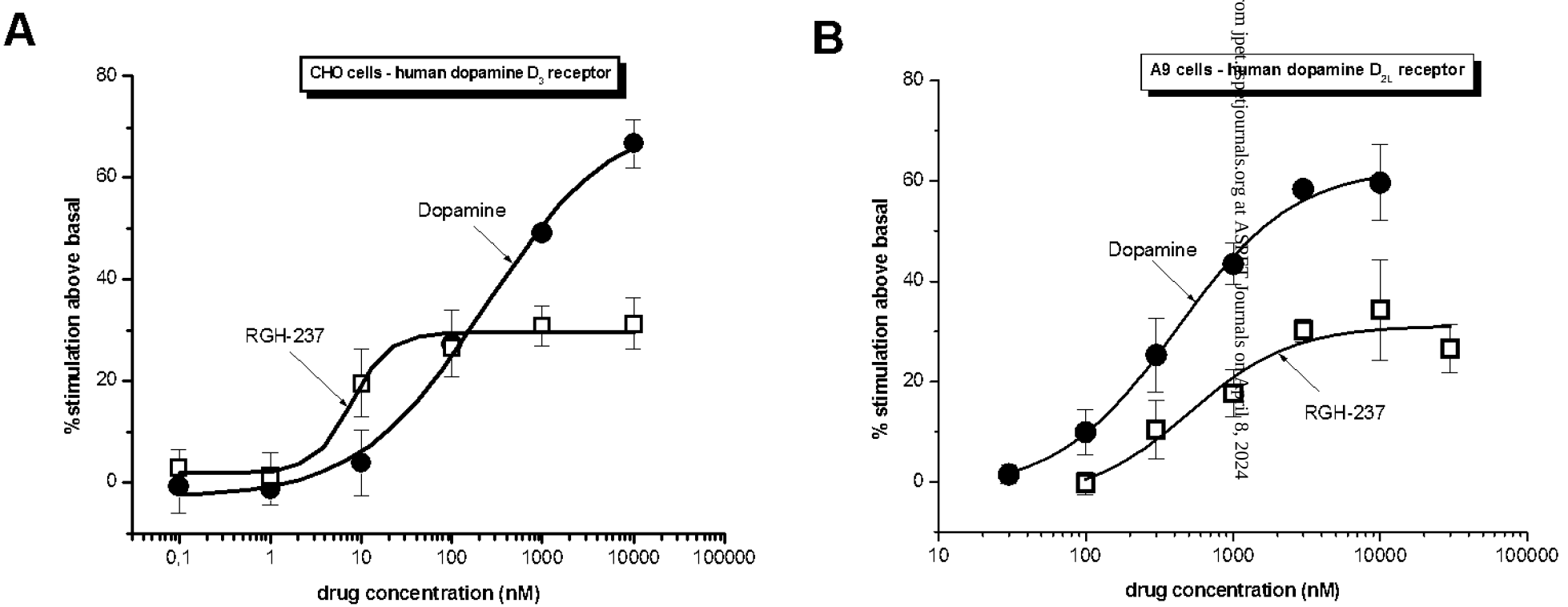


Fig. 3

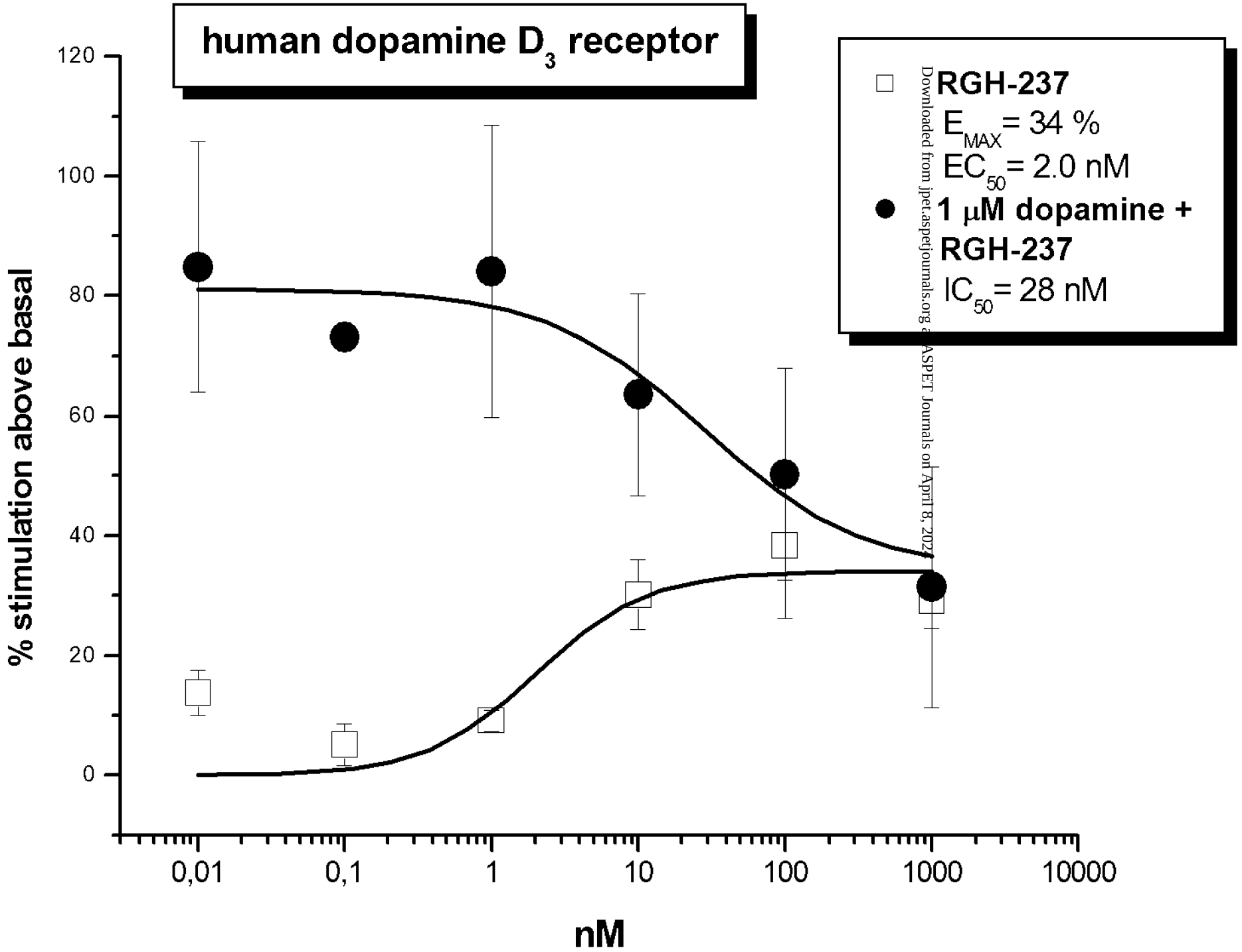
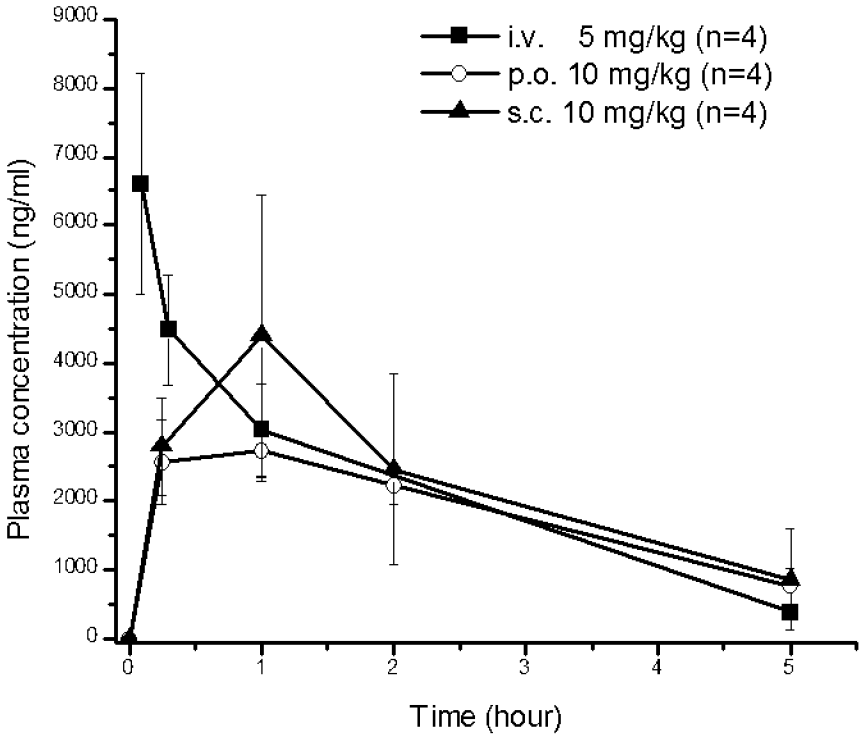


Fig. 4



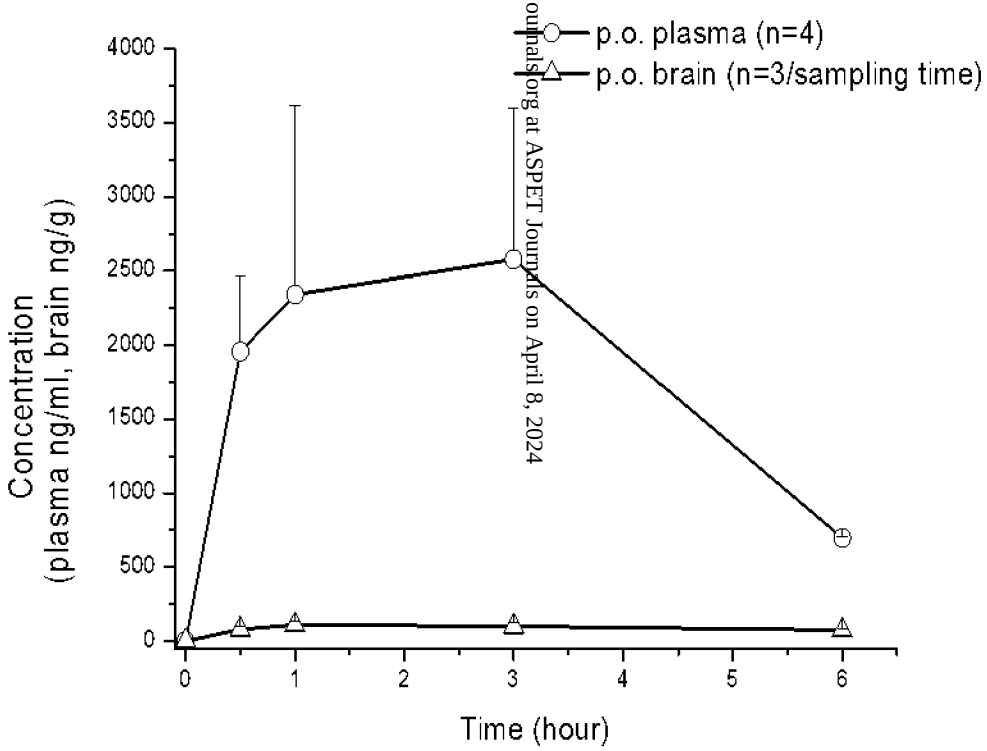


Fig. 5

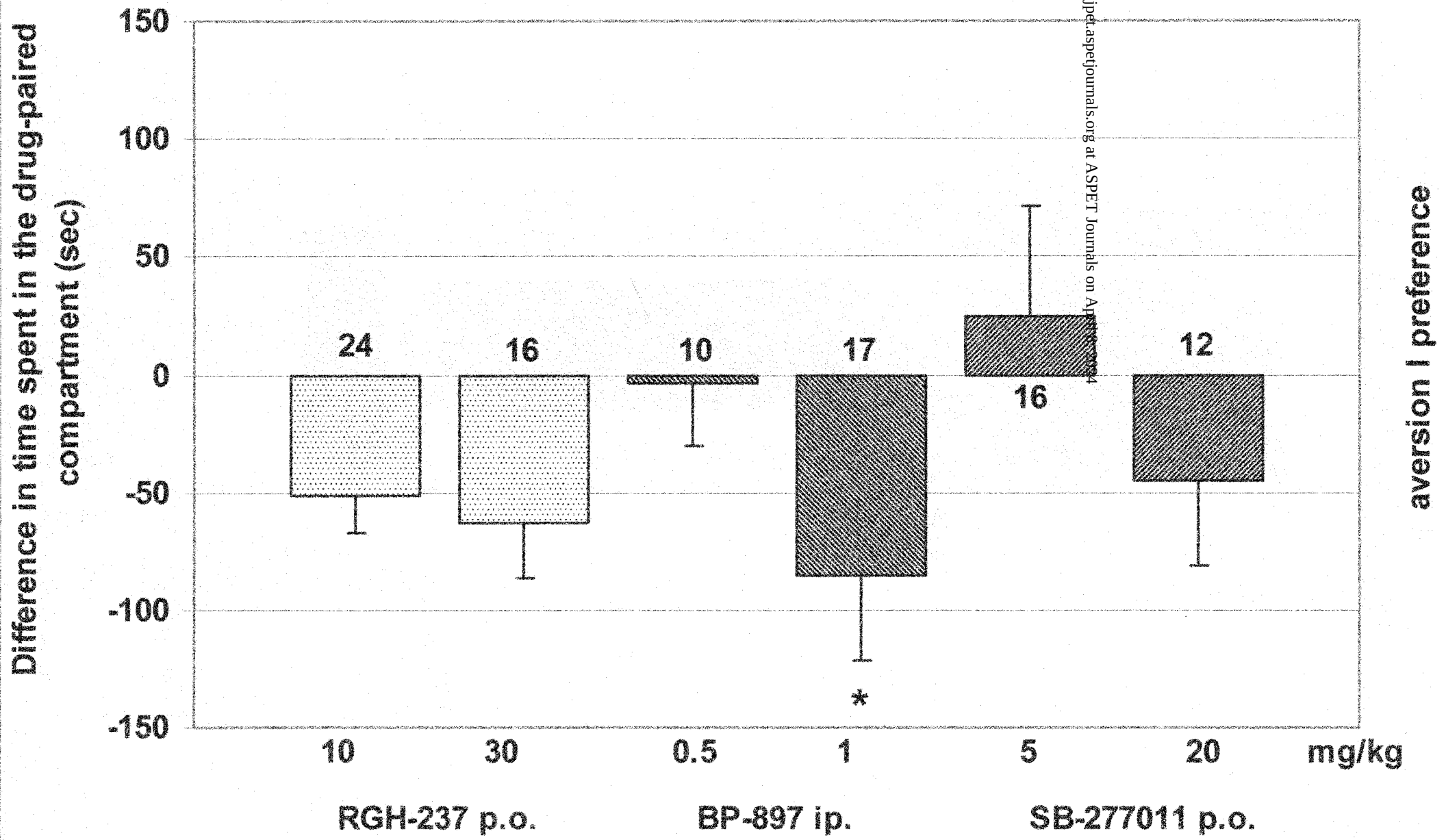


Fig. 6

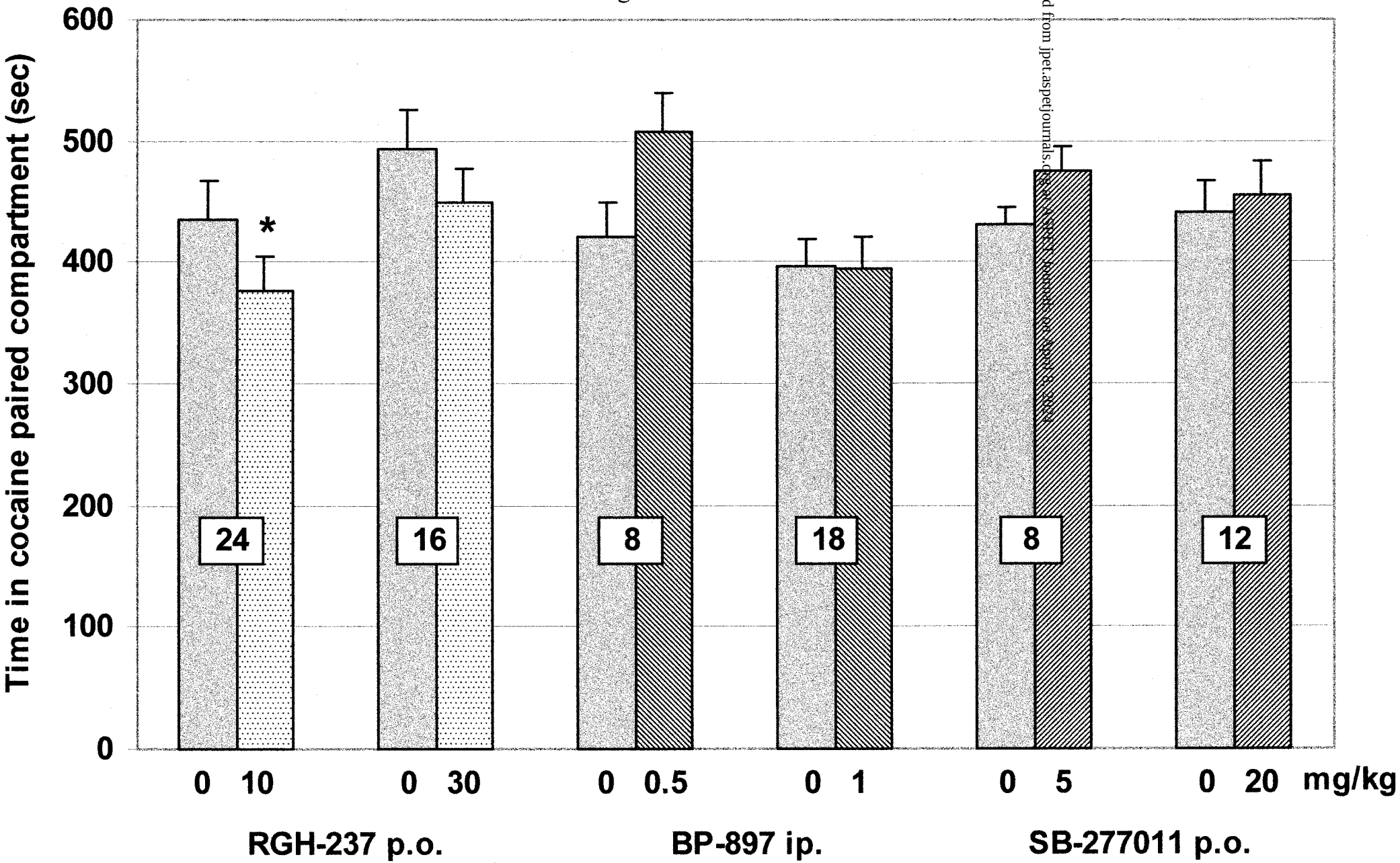


Fig. 7

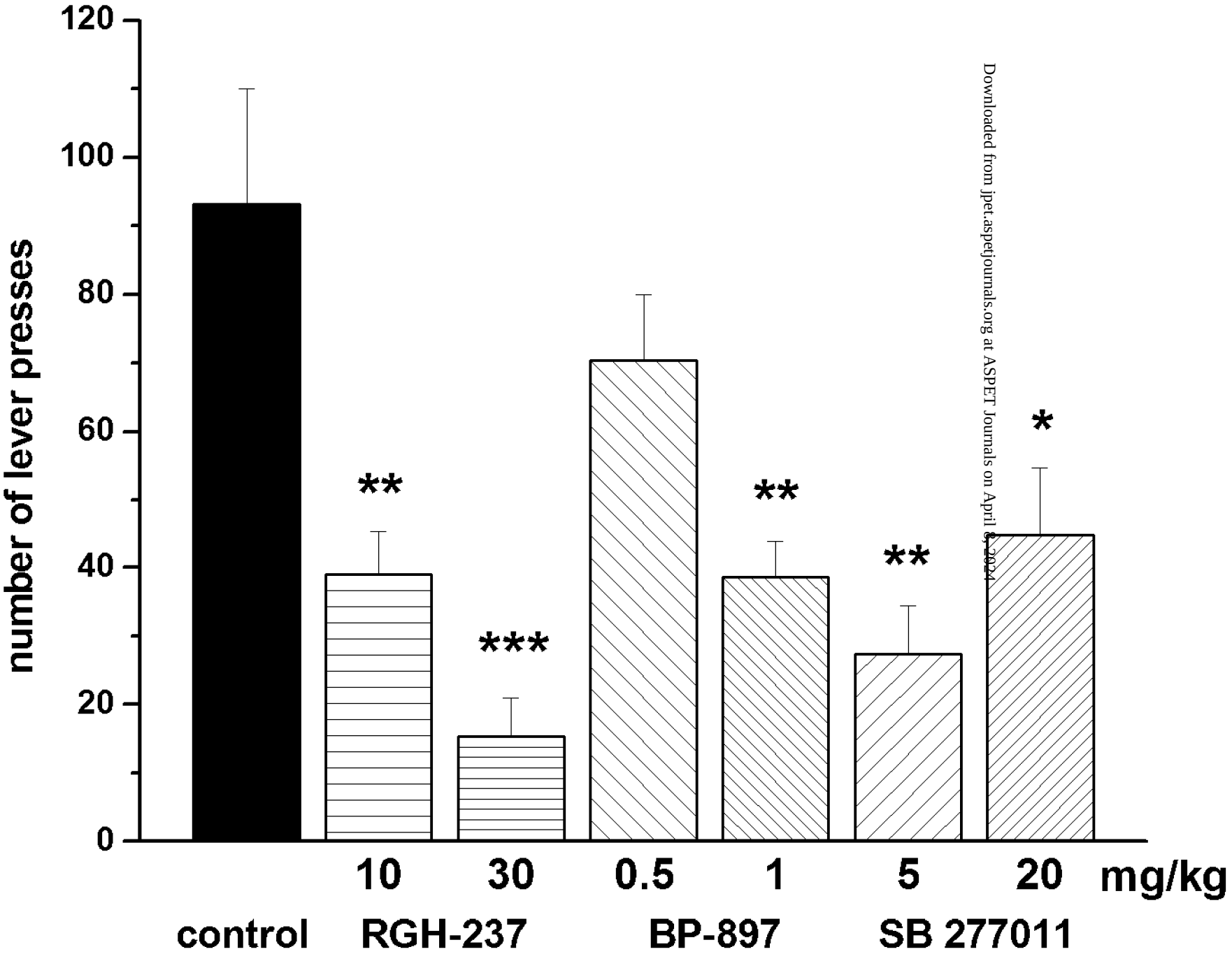


Fig. 8

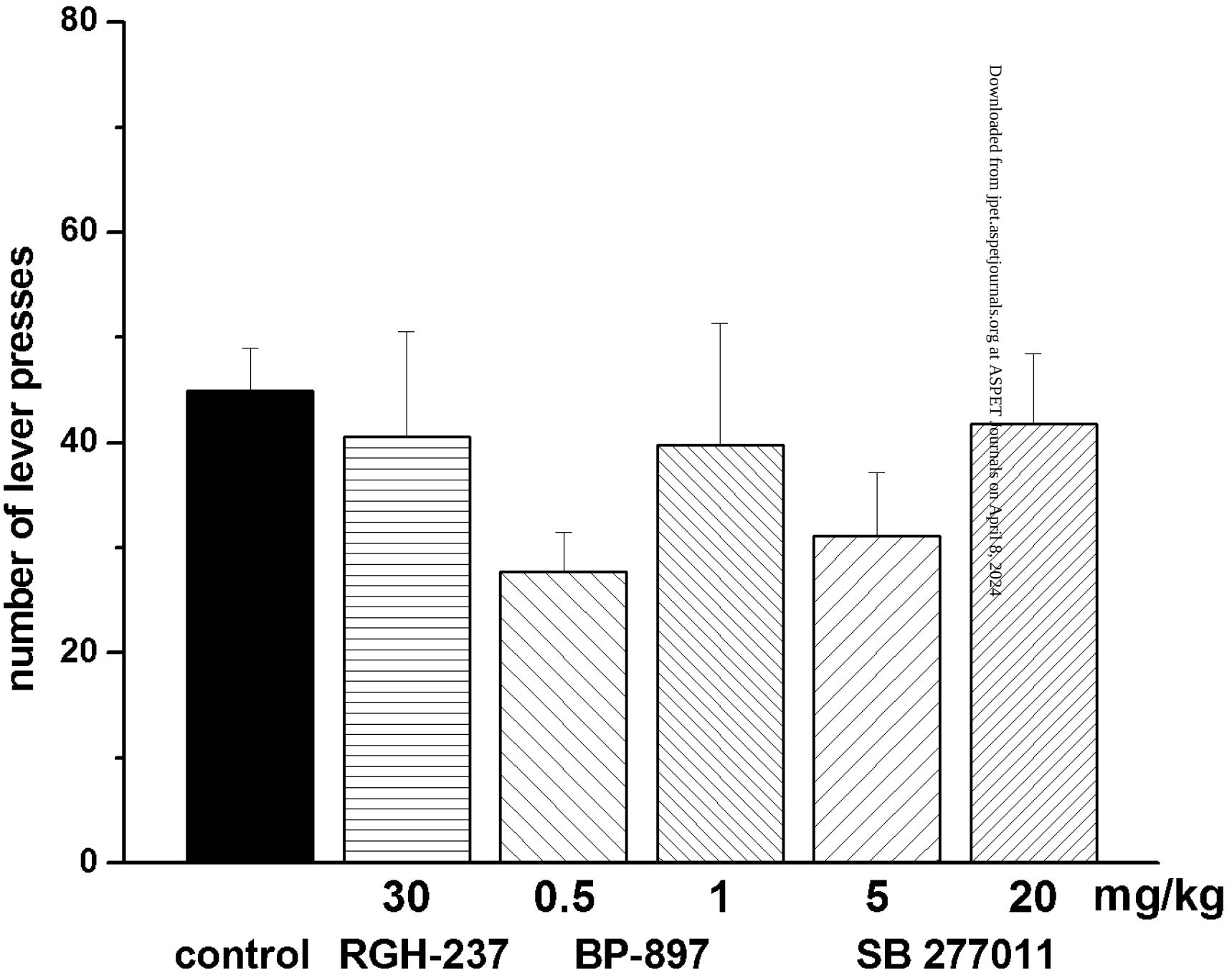


Fig. 9