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The NR2B-selective NMDA receptor antagonist Ro 25-6981 potentiates the effect of nicotine on locomotor activity and dopamine release in the nucleus accumbens

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Ro 25-6981 potentiates the effect of nicotine

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D) Abbreviations: Ro 25-6981, (+/-)-(R*, S*)-alpha-(4-hydroxyphenyl)-beta-methyl-4-(phenylmethyl)-1-piperidine propanol; VTA, ventral tegmental area; NAcc, nucleus accumbens; PFC, prefrontal cortex; CGS19755, cis-4-phosphonomethyl-2-piperidine carboxylic acid; AP-5, 2-amino-5-phosphopentanoic acid; NMDA, N-methyl-D-aspartate; CGP39551, (*E*)-(±)-2-Amino-4-methyl-5-phosphono-3-pentenoic acid ethyl ester; LMA, locomotor activity; DA, dopamine; nAch, nicotinic acetylcholine; 5-HT, 5-hydroxytryptamine; NA, noradrenaline; GABA, γ-amino-butyric acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate

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Abstract

It has been proposed that nicotine-stimulated locomotor activity (LMA) and nicotine-induced dopamine (DA) release in the mesocorticolimbic DA system is partly regulated by glutamate receptors, particularly *N*-methyl-D-aspartate (NMDA) receptors. The functional characteristics of NMDA receptors depend on their subunit composition (NR1 in combination with NR2A-D). In the present study, we investigated the effect of the NR2B-selective NMDA receptor antagonist Ro 25-6981 on nicotine-stimulated LMA and nicotine-induced DA release in the nucleus accumbens (NAcc) in rats. Ro 25-6981 (3 and 10 mg/kg, i.p.) given 10 minutes prior to a high dose (0.6 mg/kg, s.c.) or a subthreshold dose (0.1 mg/kg, s.c.) of nicotine potentiated nicotine-stimulated LMA with no effect when administered alone. Similarly, administration of a low dose (0.05 mg/kg, i.p.) of the non-competitive NMDA receptor antagonist MK-801 had no effect on LMA by itself but potentiated nicotine-induced (0.1 mg/kg) LMA. However, pretreatment with the competitive NMDA receptor antagonist CGP39551 (10 mg/kg, i.p.) did not potentiate the LMA effect of 0.1 mg/kg nicotine as seen with Ro 25-6981. *In vivo* microdialysis revealed a significant increase of DA release in the NAcc in response to nicotine (0.1 mg/kg, s.c.). In analogy to our LMA data, Ro 25-6981 (10 mg/kg, i.p.) significantly potentiated the nicotine-induced DA release although it had no effect on DA-release when given alone. The data suggests that, compared to other subunits of the NMDA receptor, the NR2B subunit might play a different role in the reinforcing effects of nicotine.

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Nicotine is considered to be the major psychoactive and dependence-producing substance in tobacco products (Picciotto and Corrigall, 2002). The reinforcing effect of nicotine is thought to be a result of an increase in dopamine (DA) release in the mesolimbic pathway which projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) (Imperato et al., 1986; Di Chiara, 2000). Regardless of whether nicotine is administered systemically or locally into the VTA or NAcc, it binds to nicotinic acetylcholine (nAch) receptors located on dopaminergic neurons, depolarizes them and increases DA release in the NAcc (Mifsud et al., 1989; Nisell et al., 1994; Fu et al., 2000). Besides dopamine, systemically administered nicotine has also been shown to alter the release of many other neurotransmitters including endogenous opioids, noradrenaline (NA), 5-hydroxytryptamine (5-HT), γ-amino-butyric acid (GABA), and glutamate (Watkins et al., 2000).

Experimental evidence indicates that nicotine facilitates glutamatergic transmission in the frontal cortex, NAcc, and VTA and glutamatergic transmission is suggested to modulate DA transmission in the mesocorticolimbic pathway (Toth et al., 1993; Fu et al., 2000; Reid et al., 2000). Indeed, the recent observation that intrategmental injection of the competitive glutamate N-methyl-D-aspartate (NMDA) receptor antagonists 2-amino-5-phosphopentanoic acid (AP-5) or cis-4-phosphonomethyl-2-piperidine carboxylic acid (CGS19755) blocks nicotine-induced DA release in the NAcc, have led to the hypothesis that nicotine may influence the release of DA via pre-synaptic modulation of the excitatory glutamatergic input to the neurons in the VTA (Schilstrom et al., 1998; Fu et al., 2000; Mansvelder and McGehee, 2000). Glutamate binds to three classes of ionotropic glutamate receptors; α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA), kainate, and NMDA receptors (Dingledine et al., 1999). Functional NMDA receptors are heteromeric complexes

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assembled from NR1 and NR2 subunits. The NR1 subunits are formed from a single gene product with 8 splice variants whereas NR2 subunits form from four different gene products; NR2A, NR2B, NR2C, and NR2D (Hollmann and Heinemann, 1994). The NMDA receptors show distinct distribution patterns in the adult rat brain compared to the developing brain, suggesting that there might be different populations of neurons with unique NMDA receptor subunit compositions and distinct pharmacological properties (Monyer et al., 1994; Monaghan and Larsen, 1997). For instance, in both non-human primates and rats, NR2B-subunits are highly expressed in the cerebral cortex (in particular in pyramidal like cells in layer II/III and V). Also, NR2B-subunits are highly abundant in the thalamus, all neurons in the striatum, in the fields of Ammon´s horn and the CA1, CA3, and dentate gyrus of the hippocampus (Loftis and Janowsky, 2003). Importantly, the VTA seems to contain NMDA receptors with a high proportion of the NR2B subunit compared to NR2A, NR2C and NR2D (Allgaier et al., 1999). Based upon those observations and given the fact that we (Kosowski et al., 2004) recently demonstrated that systemic administration of the specific but non-selective NMDA receptor antagonist CGP39551 blocked nicotine-induced increase of LMA and DA release in the NAcc, we wanted to investigate whether this effect was mediated through the NR2B-subunit.

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Materials and Methods

Animals

All experiments were approved by the Ethical Committee for Use of Animal Subjects at Karolinska Institutet and carried out in compliance with local animal care guidelines and National Institute of Health's Guide for the Care and Use of Laboratory Animals. A total of 328 male Wistar rats (Scanbur BK AB, Sollentuna, Sweden) weighing 290 g ± 20 g at the start of the experiment were allowed free access to standard rat chow and water. Upon arrival to the animal facility, animals were housed in groups of four in a temperature- (22C°) and humidity- (50%) controlled environment on a 12-h light/dark cycle (lights on at 7 am). Before the start of the experiments the animals were left for 7 days to ensure acclimatization.

Drugs

(-)-Nicotine hydrogen tartrate salt (Sigma-Aldrich St.Louis, MO, USA) was dissolved in saline (0.9%), pH- adjusted to 7.2 ± 0.2 with [1M] NaOH and administered at doses ranging from 0.05 to 0.6 mg/kg (s.c.) free base in a volume of 1 ml/kg. MK-801 (Sigma-Aldrich St.Louis, MO, USA) was dissolved in distilled water and administered in doses of 0.02, 0.05, 0.15, and 0.3 mg/kg (i.p.) in a volume of 1 ml/kg (hydrogen maleate form). CGP39551 (a generous gift from Novartis, Basel, Switzerland and purchased from Tocris Cookson Ltd, MO, USA) was dissolved in saline and administered at 1, 3, or 10 mg/kg (i.p) in a volume of 2 ml/kg. Ro 25-6981 (Tocris Cookson Ltd, MO, USA) was dissolved in saline and administered in doses of 1.0,

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3.0, and 10 mg/kg (i.p.) in a volume of 5 ml/kg. [3H]Epibatidine (56.2 Ci/mmol) was purchased from PerkinElmer Life Sciences, Inc. Boston, MA, USA). Pentobarbital sodium and bupivacaine (Apoteket AB, Stockholm, Sweden) were given at 60 mg/kg i.p. and 0.3 ml, respectively

Locomotor activity

LMA was measured using four AccuScan activity meters (42x42x30 cm) (AccuScan Instruments Inc, Ohio, USA) equipped with three rows of infrared photo sensors. Each row consisted of 16 sensors, 2.5 cm apart where two rows were placed around the bottom and the third row was placed 10 cm above the floor to measure vertical activity. All measurements were conducted (according to a between-subject experimental design) between 8 am and 5 pm and rats (n=292) were habituated to the LMA boxes for two days before any drug treatment commenced. Each time a photo beam was crossed, it was recorded as one activity count. During the first day of habituation, the rats were allowed to freely explore the LMA boxes for one hour. The second day of habituation was designed to habituate the rats to the injection and to simulate the test situation. The rats were allowed to freely explore the activity boxes for 30 minutes and were then given an injection of saline (1 ml/kg, s.c.). Following the saline injection, the rats were returned to the activity boxes and allowed an additional period of 60 minutes for free exploration. On the third day, rats were placed in the activity boxes and given 30 minutes to reduce possible transport-induced stress and were then administered either saline, MK-801, CGP39551 or Ro 25-6981. Ten minutes later, either saline or nicotine was given and LMA was recorded for 60 minutes. Behavior was recorded with digital video cameras

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(Panasonic, NV-DS27EG) set up in front of each activity cage. The behavior of the rats was rated once every 5 minutes for 30 minutes using a 9-point scale developed by Ellinwood and Balster (Ellinwood and Balster, 1974). Scores ranging from 1 to 4 define normal activity behavior from asleep, score 1, to running around, sniffing and rearing, score 4. Stereotypy scores ranging from 5 to 9 define increased severity of stereotypic behavior where score 5 represents hyperactive movements with jerky moves and score 9 is characterized by seizures, abnormally maintained postures and dyskinesias.

Microdialysis

The microdialysis procedure and high performance liquid chromatography (HPLC) electrochemical analysis have been described previously (Kosowski et al., 2004). Briefly, guide cannulas for the probe (CMA 12, CMA Microdialysis, Stockholm, Sweden) were implanted above the NAcc [AP: 1.6, ML: 1.2, and DV: -8.0, according to the brain atlas of Paxinos and Watson, (Paxinos and Watson, 1986)]. At the day of experiment, animals (n=27) were transferred to a separate room and microdialysis probes (CMA12/2 mm) were inserted and perfused with artificial cerebrospinal fluid. Two hours following probe insertion; six consecutive samples were collected to measure basal levels of DA before any drug treatment. Each sample was collected over 10 minutes at a flow rate of 2 μl/min in vials prefilled with 0.3 M perchloric acid and a total of 18 samples were collected. At the end of the experiment rats were intracardially perfused with PBS and 4% paraformaldehyde. Brains were removed and stored in 30% PBS/sucrose solution, cryosectioned at 25 μm and stained with thionin. Probe position was determined histologically according to the brain atlas of

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Paxinos and Watson, 1986. Only data obtained from animals with probes correctly placed within the NAcc were used in the analysis. A probe was considered to be correctly located when it transversed the mediodorsal core and ventral shell.

Analysis of dialysate

The concentration of DA was analyzed with reverse phase HPLC systems (ESA Inc., Chelmsford, MA, USA) with electrochemical detection using a Coulochem II detector (5200A) with a conditioning cell (5021) and an analytical cell (5011) where one of the systems only had the analytical cell. The mobile phase, (Na-acetate; 7.465 mg/l, Na_2EDTA; 3.7 mg/l, octanesulfonic acid monohydrate; 140.79 mg/l, and HPLC-graded methanol; 110 ml/l and pH adjusted to 4.1 with concentrated acetic acid) was delivered by an HPLC-pump (Model 582, ESA Inc., Chelmsford, MA, USA) through a C18-AQ column (Reprosil-Pur, 150X4 mm, 5μ) at a flow rate of 1 ml/min. Samples (25 μl) were automatically injected by an autosampler (Model 830, Midas, Spark Holland BV). The oxidizing potentials were set as follows; HPLC-system 1: conditioning cell: +175 mV, analytical cell R2 +400 mV and HPLC-system 2: analytical cell R1 +75mV, R2 +350 mV. The microdialysis samples were randomly assigned to one of the two HPLC systems; all samples from a given subject were analyzed with the same system. Chromatographic analysis was performed using CSW 1.7 software (DataApex Ltd, Czech Republic).

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Nicotine receptor binding assay

Tissue preparation

The binding assay has been described previously (Kosowski et al., 2004). Briefly, rats were sacrificed by decapitation and the brains were removed and the cerebral cortex was dissected on an ice-cold glass plate and stored at -80ºC until use. The tissue was homogenized in an ice-cold hypotonic buffer solution (0.1 x HEPES buffer: 118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM HEPES, pH 7.4, 1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride, 0.02% w/v sodium azide) using a Polytron homogenizer (10 s at setting 5) (Kinematica, Switzerland). A total of 9 cortices were pooled to acquire enough tissue for the binding assays and the crude particulate fraction was obtained by centrifugation at 15 000 rpm for 15 min at 4ºC. The supernatant was discarded and the pellet was washed twice more by resuspension in ice-cold homogenization buffer using a glass-teflon tissue grinder and centrifugation. The final pellet was stored at -80ºC until use.

Binding assay

The pellet was resuspended in HEPES buffer (pH 7.4) and the protein concentration was measured using a Bio-Rad Protein assay with bovine serum albumin as the standard. Aliquots of tissue homogenates (≈0.2 mg of protein) in triplicates were incubated in polypropylene test tubes in 50 mM Tris-HEPES buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM HEPES, pH 7.4) containing 100 pM [^{3}H]Epibatidine. The concentration of [3H]Epibatidine was chosen based on a

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previously determined saturation binding curve which had a K_d of 10.4 ± 1.4 pM (data not shown) where also non-specific binding was determined in the presence of 100 µM (-)-nicotine hydrogen tartrate. Increasing concentrations of Ro 25-6981 or nicotine (1 nM to 100 µM) were added to the polypropylene test tubes. Final assay volumes amounted to 5 ml to avoid ligand depletion. The reaction was started by the addition of tissue and the mixture was incubated for two hours at room temperature in the dark. Incubation was terminated by vacuum filtration through Whatman GF/C filter (presoaked in binding buffer containing 0.5% polyethyleneimine) using a Brandel cell harvester (Gaithersburg, MD, USA). The filter was rapidly washed four times with 4 ml aliquots of cold 50 mM Tris-HCl buffer (pH 7.4). Subsequently, filters were placed in scintillation vials with 5 ml Ready Safe liquid scintillation cocktail (Beckman Coulter, CA, USA) and counted in a β-counter (Wallac, Finland) at 45% counting efficiency. The IC_{50}-value was calculated from the curve in the graph, which was fitted by nonlinear regression analysis (GraphPad Prism, GraphPad Software, San Diego, CA, USA).

Data analysis

LMA data are presented as mean (± SEM) total horizontal activity counts over 60 minutes and analyzed using one-way ANOVA followed by Bonferroni´s or Dunnett's test for multiple comparisons when appropriate. Alternatively, data are presented as mean (± SEM) total activity counts per 5 minutes over the hour after the last drug administration and analyzed using 2-way ANOVA (treatment x time) with repeated measures followed by Bonferroni´s test for multiple comparison when appropriate.

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DA levels are expressed as percent of baseline, which was defined as the average of the three samples immediately preceding treatment. The mean percent changes were then calculated for each 10-minute sample for all rats in each group. Data were analyzed statistically with 2-way ANOVA (treatment x time) with repeated measures followed by Bonferroni´s test for multiple comparisons when appropriate.

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Results

Locomotor activity

Effect of Ro 25-6981 and nicotine on locomotor activity

Systemic administration of nicotine elicited a dose-dependent locomotor stimulant response (Fig 1). The threshold dose for LMA was 0.2 mg/kg (3915 ± 645 activity counts) and doses of 0.3 and 0.6 mg/kg significantly increased LMA (4679 ± 573 and 5785 ± 385 activity counts respectively, $F_{(5, 36)}$ = 7.00, P<0.0001, n = 7). Thus, the dose of 0.1 mg/kg (2750 ± 520 activity counts) was chosen as a representative subthreshold dose of nicotine. Ro 25-6981 (1.0, 3.0, and 10 mg/kg) did not alter spontaneous, basal LMA ($F_{3,28}$ = 0.85, P = 0.48, n = 8), (Fig. 2). When administered 10 minutes prior to nicotine (0.6 mg/kg, s.c. free base), Ro 25-6981 (3.0 and 10 mg/kg, i.p.) enhanced nicotine-stimulated locomotor activity ($F_{3,40}$ = 13.55, P<0.0001), (Fig. 3). This effect was only seen at the highest dose of Ro 25-6981 (10 mg/kg), which significantly increased total horizontal activity (9336 ± 1249 activity counts) as compared to nicotine treated animals (4830 ± 549.8 activity counts), (p<0.01, n = 11). Since the originally used dose of nicotine (0.6 mg/kg) significantly increased LMA by itself, it was difficult to determine whether the enhanced LMA evoked by Ro 25-6981 should be regarded as an additive or potentiating effect. To clarify this issue, Ro 25-6981 (10 mg/kg) was administered prior to a subthreshold dose of nicotine (0.1 mg/kg) (Fig 4a). The result was a significant increase in LMA, (main effect of $F_{2,18}$ = 7.2, P = 0.0051, n = 7). A post hoc analysis verified that the combined treatment with Ro 25-6981 (10 mg/kg) and nicotine (0.1 mg/kg) indeed significantly potentiated LMA (p<0.05, n = 7). The increase in LMA appears to occur

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during the initial 30 minutes after nicotine administration (Fig. 4b) since the post-hoc test of a two-way ANOVA (a significant main effect ($F(treatment)_{1,13} = 12.42$, $P<0.0001$) was significant at 25 and 30 minutes ($p<0.05$ and $p<0.01$, respectively). The video analysis showed no observable drug-induced stereotypic behavior.

Effects of CGP39551 and nicotine on locomotor activity

An additional series of experiments were conducted to investigate the effect of competitive NMDA receptor antagonist, CGP39551, on LMA when given prior to the subthreshold dose of nicotine. The dose-response curve in Fig. 5 shows that CGP39551 (1, 3, and 10 mg/kg) had no effect on spontaneous LMA alone ($F_{3.28} = 0.4$, $P = 0.76$, $n = 8$). Furthermore, CGP39551 (10 mg/kg), administered 10 minutes before nicotine (0.1 mg/kg) did not change LMA compared to saline-treated control animals ($F_{3,32} = 0.55$, $n = 9$), (Fig. 6).

Effects of MK-801 and nicotine on locomotor activity

Administration of the non-competitive NMDA receptor antagonist MK-801 stimulated LMA (Fig. 7). A one-way ANOVA shows that MK-801 significantly increased LMA ($F_{4,25} = 16.44$, $P<0.0001$) and Dunnett's post hoc test revealed that the two highest doses given (0.15 and 0.3 mg/kg, 6223 ± 767 and 13630 ± 2836 activity counts, respectively, $p<0.05$ and $p<0.01$) significantly increased LMA compared to saline treated control animals (2849 ± 395 activity counts, $n = 6$). However, the highest dose of MK-801 produced abnormal locomotor activity with a high incidence of stereotypies (data not shown). Based on the dose-response curve in Fig. 7, MK-801, at doses with no effect on LMA *per se* (0.02 and 0.05 mg/kg), was administered 10 minutes prior to nicotine (0.1 mg/kg), (Fig. 8). Only MK-801 (0.05 mg/kg, i.p.) followed

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by nicotine significantly potentiated LMA, ($p<0.05$), (Sal + Nic 0.1: 4001 ± 606 vs MK-801 0.05 + Nic 0.1: 7626 ±1214 activity counts), (n=8).

Microdialysis

Figure 9 depicts the placement of the microdialysis probes in the NAcc. In analogy with the LMA results, Ro 25-6981 (10 mg/kg) administered 10 minutes prior to nicotine (0.1 mg/kg) significantly enhanced DA release in the NAcc ($F(\text{treatment})_{1,13}$ = 17.35, $P<0.0001$), (Fig 10). The mean baseline level of DA in the NAcc dialysates was 10.38 ± 0.59 fmol/10 µl (n = 6-7). When administered alone, Ro 25-6981 (10 mg/kg) did not alter basal DA release in the NAcc. However, in contrast to the LMA results shown in Fig. 4, nicotine (0.1 mg/kg) significantly increased DA release in the NAcc ($F(\text{treatment})_{1,13}$ = 34.61, $P<0.0001$). A maximal level of DA release was reached 20 minutes after administration of nicotine (155 %), which then slowly declined until reaching the initial baseline levels 100 minutes later (see Fig. 10). Pretreatment with Ro 25-6981 (10 mg/kg) resulted in a more pronounced and long lasting DA release compared to nicotine-treated animals and reached a peak level (182%) 30 minutes after nicotine injection. Compared to the LMA data where the increase was seen during the initial 30 minutes, the DA levels remained significantly elevated (141%) until the last microdialysis sample (collected 100 minutes after the administration of nicotine).

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Nicotine receptor binding assay

Some studies have provided evidence that MK-801 has affinity to nicotinic receptors. To address the question of whether Ro 25-6981 also possesses nicotine receptor binding properties, a radio-ligand binding assay was performed. As can be seen in Fig. 11, (-)-nicotine hydrogen tartrate effectively inhibited binding of 100 pM [3H]Epibatidine in a concentration-dependent manner (1 pM to 100 μM). In contrast, increasing concentrations of Ro 25-6981 up to 100 μM did not inhibit [^{3}H]Epibatidine. The IC_{50} value for (-)-nicotine hydrogen tartrate was 7.2 nM whereas no binding at any concentration was observed for Ro 25-6981.

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Discussion

In the present study, the most interesting finding is that the NR2B-selective NMDA receptor antagonist Ro 25-6981 potentiated LMA to a subthreshold dose of nicotine and also enhanced nicotine-induced DA release in the NAcc. Recent reports indicate that blockade of glutamatergic transmission with NMDA receptor antagonists administered systemically or intrategmentally can inhibit NAcc DA release or LMA elicited by nicotine (Schilstrom et al., 1998; Sziraki et al., 1998; Laviolette and van der Kooy, 2003). However, many of the early NMDA receptor antagonists (e.g. the non-competitive antagonist MK-801) produced adverse psychotomimetic effects (Svensson, 2000). Significant progress in the understanding of the NMDA receptor complex has led to the development of novel compounds that seem to produce less side effects; such as the NR2B-selective antagonists ifenprodil and Ro 25-6981 (Williams, 1993; Fischer et al., 1997; Mutel et al., 1998). In contrast to Ro 25-6981, ifenprodil is not fully selective since it also binds with high affinity to other classes of receptors (α_1-, 5HT1α-, and σ-receptors). Since intrategmental infusions of NMDA receptor antagonists block nicotine-induced NAcc DA release and the VTA contains a high proportion of NMDA receptor NR2B-subunits, we hypothesized that systemic administration of Ro 25-6981 would block nicotine-stimulated LMA and nicotine-induced DA release in the NAcc. Surprisingly, Ro 25-6981 enhanced the LMA stimulatory actions of nicotine without producing any overt behavioral changes. This enhanced locomotor stimulation was in line with previously observed effects following administration of for example MK-801 (Shoaib et al., 1994). A complicating factor in this context is that administration of MK-801 alone stimulates LMA and induces stereotypic behavior in rats and mice as also verified in this study (Liljequist, 1991;

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Svensson et al., 1998). In addition, administration of a subthreshold dose of MK-801 (0.05 mg/kg) prior to nicotine (0.1 mg/kg) potentiated LMA. MK-801 and Ro 25-6981 therefore seem to exert similar effects on LMA when co-administered with nicotine but differ when each of the compounds is given alone. An additional series of experiments were conducted to investigate the influence of CGP39551, a competitive NMDA receptor antagonist. Consequently, when administered alone or 10 minutes prior to nicotine (0.1 mg/kg), CGP39551 [10 mg/kg, a dose previously shown to block nicotine-stimulated LMA (Kosowski et al., 2004)] did not affect LMA. Thus, in contrast to Ro 25-6981 and MK-801, CGP39551 does not potentiate LMA when given together with nicotine (0.1 mg/kg) although it, like Ro 25-6981, seems to lack the LMA stimulating properties displayed by MK-801.

In agreement with current LMA results pretreatment with Ro 25-6981 (10 mg/kg) enhanced nicotine-induced DA release in the NAcc but did not alter basal DA release by itself. In this context, it is important to note that, in analogy with Ro 25-6981, competitive NMDA receptor antagonists that bind reversibly to the NMDA receptor do not seem to influence DA release in NAcc in rats when given alone at anticonvulsive doses (Kretschmer, 1999). However, in contrast to Ro 25-6981, they have the property to effectively inhibit nicotine-induced DA release in the NAcc (Fu et al., 2000). On the other hand, non-competitive antagonists (such as MK-801), which inhibit nicotine-induced DA release in the NAcc by blocking the ion channel of the NMDA receptor, increase DA release in the NAcc when administered alone at anticonvulsive and anti-cataleptic doses (Kretschmer, 1999). Thus, when compared to MK-801 and CGP39551, Ro 25-6981 clearly displays a different mode of action. The effect of MK-801 on NAcc DA release and LMA seems to depend on the activity of DA neurons in the VTA as systemically administered MK-801 and other non-

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competitive NMDA receptor antagonists increase the firing rate and burst firing of these neurons (French et al., 1993). Another complicating factor is that MK-801 also appears to be an antagonist of brain nicotinic receptors (Buisson and Bertrand, 1998). The possibility that Ro 25-6981 possesses affinity to nicotinic receptors seems, however, unlikely since the binding assay with [3H]Epibatidine revealed no affinity for nicotinic binding sites.

A common feature of drugs of abuse, including nicotine, is that they increase NAcc DA release as well as stimulate LMA (Wise and Bozarth, 1987). However, the results in the present study showed that a dose of nicotine with no effect on LMA significantly increases NAcc DA release. A possible explanation for this discrepancy might be the location of the microdialysis probes. Acute nicotine has been shown to increase DA more robustly in the shell subregion of the NAcc compared to the core subregion (Cadoni and Di Chiara, 2000). Also, nicotine, at a dose that significantly increases DA in the ventral striatum only modestly increases DA in dorsal striatum (Seppa and Ahtee, 2000). Importantly, the shell subregion is intimately connected with limbic structures while the core subregion and the dorsal striatum are more extensively connected with motor circuits (Kalivas and McFarland, 2003). Since the microdialysis probes were located predominantly in the ventral shell but also in the mediodorsal core, the released DA that was microdialyzed originated from the areas associated with the limbic system. Consequently, it could be hypothesized that the dose of nicotine able to activate DA release in the limbic part of the NAcc was too low to activate the motor circuits and thus not able to affect LMA.

Moreover, there was an interesting dissociation of the effects of the combination of Ro 25-6981 and nicotine in terms of the increase in magnitude and prolongation of effects on DA release and LMA. Compared to nicotine alone, the combined treatment

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of Ro 25-6981 and nicotine increased the amount of released DA and also the duration of this release. However, Ro 25-6981 and nicotine together only increased the total amount of LMA but not the duration of LMA stimulation. Since all drugs were administered systemically, it is difficult to speculate what mechanisms might be involved. Nicotine has been shown to induce a long-lasting release of NAcc DA release, with an initial peak in DA levels that declines but remains elevated for at least two hours after nicotine administration (Di Chiara, 2000). This effect has been attributed to the actions of two nicotinic receptor subtypes. Initially, nicotine binds to $\alpha_4\beta_2$-receptors located post-synaptically on dopaminergic cells in the VTA, depolarizes them and ultimately increases DA release in the NAcc. This event corresponds to the peak in DA levels. Subsequently, the $\alpha_4\beta_2$-receptors desensitize and become inactive. The prolonged elevation in NAcc DA levels is thought to arise from nicotine binding to pre-synaptic α_7-receptors located on afferents in the VTA projecting from the prefrontal cortex (PFC). Upon binding, glutamate is released and binds to NMDA receptors on dopaminergic cells in the VTA and elicits burst firing which increases the release of NAcc DA, [for review see (Dani et al., 2001)]. In analogy to ifenprodil, Ro 25-6981 seems to antagonize NMDA receptors in an unusual activity-dependent manner (Mutel et al., 1998). Thus, both ifenprodil and Ro 25-6981 increase the affinity of NMDA receptors in the presence of low concentrations of NMDA (inactive state) and this enhances the effects of NMDA receptor agonists. However, as the NMDA concentration increases (moving towards the active state), the affinity-enhancing effect of these two compounds decrease and they start to function as NMDA receptor antagonists (Kew et al., 1996; Zhang et al., 2000). Therefore, the potentiation of LMA and DA release produced by Ro 25-6981 might be due to its modulation of NMDA receptor affinity in the NAcc and/or some

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other brain structures. Such modulation could be achieved in that nicotine stimulates glutamate to be released from pre-synaptic glutamatergic afferents and the subsequent glutamate binding to NMDA receptors located on dopaminergic neurons could give rise to a stronger response which then ultimately results in an enhanced DA release in the NAcc. This does not, however, provide an answer to why there is a difference in duration and magnitude of LMA and NAcc DA release. One simple explanation could be that the enhanced DA levels reached the threshold needed to stimulate LMA. Another more speculative explanation could be that, in analogy to nicotine-induced DA release, the length of time and amount of nicotine-stimulated LMA might depend on what specific receptor subtype nicotine acts at. Indeed, it has been shown that nicotinic receptor of the $\alpha_4\beta_2$ subtype rather than the α_7 subtype are important in mediating the expression of locomotor stimulant effects of nicotine (Kempsill and Pratt, 2000). Thus, it could be hypothesized that the prolonged release of NAcc DA, which is mediated through the α_7 subtype receptors does not necessarily imply LMA stimulation. On the other hand, given that Ro 25-6981 exerts its effects via NR2B NMDA receptors, it could interact with certain NMDA receptor populations in one or several brain regions (due to the regional distribution of NR2B subunits) and therefore produce a different net effect compared to non-selective NMDA receptor compounds.

To our knowledge, this is the first study to demonstrate that systemic administration of NR2B-selective NMDA receptor antagonist Ro 25-6981 enhanced nicotine-stimulated LMA and nicotine-induced DA-release in the NAcc. This suggests that a blockade of NMDA NR2B receptors might enhance the reinforcing and central stimulant effects of nicotine.

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Figure legends

Fig. 1. Nicotine dose-dependently increases locomotor activity. Nicotine was administered in doses ranging from 0.05 to 0.6 mg/kg (s.c. free base) and locomotor activity was measured for one hour. Data are presented as mean (±S.E.M) total horizontal activity counts and analyzed using one-way ANOVA followed by Bonferroni´s test for multiple comparisons. Point above zero represents the effect of saline injections. *p<0.05 compared to saline treated control animals (n = 7).

Fig. 2. The NR2B-selective NMDA receptor antagonist Ro 25-6981 (1, 3, and 10 mg/kg, i.p.) has no effect on spontaneous locomotor activity. Data represent mean (±S.E.M) total horizontal activity counts during one hour for each dose administered and analyzed using one-way ANOVA. Point above zero represents the effect of saline injections. No significant effects were found (n = 8).

Fig. 3. The NR2B-selective NMDA receptor antagonist Ro 25-6981 (10 mg/kg, i.p.) augments nicotine-stimulated (0.6 mg/kg, s.c. free base) locomotor activity. The data represent mean (±S.E.M) total activity counts measured over one hour after the last drug treatment and were analyzed using one-way ANOVA followed by Bonferroni´s test for multiple comparisons. ***p<0.001, **p<0.01, *p<0.05 compared to Sal + Sal treated control rats and ++p<0.01 compared to Sal + Nic 0.6 treated rats (n = 11)

Fig. 4. Administration of the NR2B-selective NMDA receptor antagonist Ro 25-6981 (10 mg/kg, i.p.) potentiates locomotor activity to a subthreshold dose of nicotine (0.1

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mg/kg, s.c. free base). Panel A represents mean (±S.E.M) total horizontal activity counts measured over one hour after nicotine administration and were analyzed with one-way ANOVA followed by Bonferroni´s test for multiple comparisons, *$p<0.05$ compared to Sal + Nic 0.1. Panel B shows a time-course presented as mean (±S.E.M) total activity counts per 5 minutes over one hour after administration of saline or nicotine (0.1 mg/kg, s.c.). Data were analyzed using two-way ANOVA with repeated measures followed by Bonferroni´s test for multiple comparisons when appropriate. **$p<0.01$, *$p<0.05$ compared to Sal + Nic 0.1 (n = 7).

Fig. 5. The competitive NMDA receptor antagonist CGP39551 (1, 3, and 10 mg/kg, i.p.) has no effect on spontaneous locomotor activity. Data represent mean (± S.E.M) total horizontal activity counts during one hour analyzed using one-way ANOVA. Point above zero represents the effect of saline injections. No significant effects were found (n = 8).

Fig. 6. The competitive NMDA-receptor antagonist CGP39551 (10mg/kg) has no effect on locomotor activity when given alone or in combination with the subthreshold dose of nicotine (0.1 mg/kg, s.c. free base). Data are presented as mean (± S.E.M) total horizontal activity counts and were analyzed using one-way ANOVA. No significant effects were found (n = 9).

Fig. 7. The non-competitive NMDA receptor antagonist MK-801 (0.15 and 0.3 mg/kg) significantly increases locomotor activity. Data are presented as mean (± S.E.M) total horizontal activity counts and were analyzed using one-way ANOVA followed by Dunnett’s post hoc test. Point above zero represents the effect of saline injections.

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**p<0.01, *p<0.05 compared to saline treated control rats (n=6).

Fig. 8. The subthreshold dose of MK-801 (0.05 mg/kg) significantly potentiates locomotor activity when given 10 minutes prior to a subthreshold dose of nicotine (0.1 mg/kg, s.c.) as compared to saline treated control. Data are presented as mean (± S.E.M.) total horizontal activity counts and were analyzed using one-way ANOVA. *p<0.05 compared to saline treated control animals (n = 8).

Fig. 9. Schematic representations of the dialysis probe placements in the NAcc. Rats were intracardially perfused with PBS and 4% paraformaldehyde. Brains were removed and stored in 30% PBS/sucrose solution**,** cryosectioned at 25 µm and stained with thionin. Probe position was determined histologically according to the brain atlas of Paxinos and Watson, 1986.

Fig. 10. The NR2B-selective NMDA receptor antagonist Ro 25-6981 (10 mg/kg, i.p.) enhances nicotine-induced (0.1 mg/kg, s.c. free base) dopamine release in the nucleus accumbens. Saline or Ro 25-6981 (10 mg/kg, i.p.) was administered at t = 0 minutes. Ten minutes later, at t = 10 minutes, saline or nicotine (0.1 mg/kg, s.c. free base) was administered and additional 11 samples were collected with 10 minute intervals. Data are presented as the mean (± S.E.M.) percent change of baseline and were analyzed using two-way ANOVA with repeated measures followed by Bonferroni´s test for multiple comparisons when appropriate. *p<0.05 compared to Sal +Nic 0.1 treatment, +++p<0.001, ++p<0.01, +p<0.05 compared to Sal + Sal treatment (n = 6-7).

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Fig. 11. In contrast to nicotine, Ro 25-6981 is unable to inhibit [3H]Epibatidine binding in rat frontal cortex. Aliquots of tissue homogenates (0.1-0.2 mg of protein) from a total of 9 rats were incubated with 100 pM [3H]Epibatidine together with the indicated concentrations of (-)-nicotine hydrogen tartrate and Ro 25-6981. Each point is the mean of 6 determinations (± S.E.M.). The curve in the graph was fitted by nonlinear regression analysis.

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Fig. 1

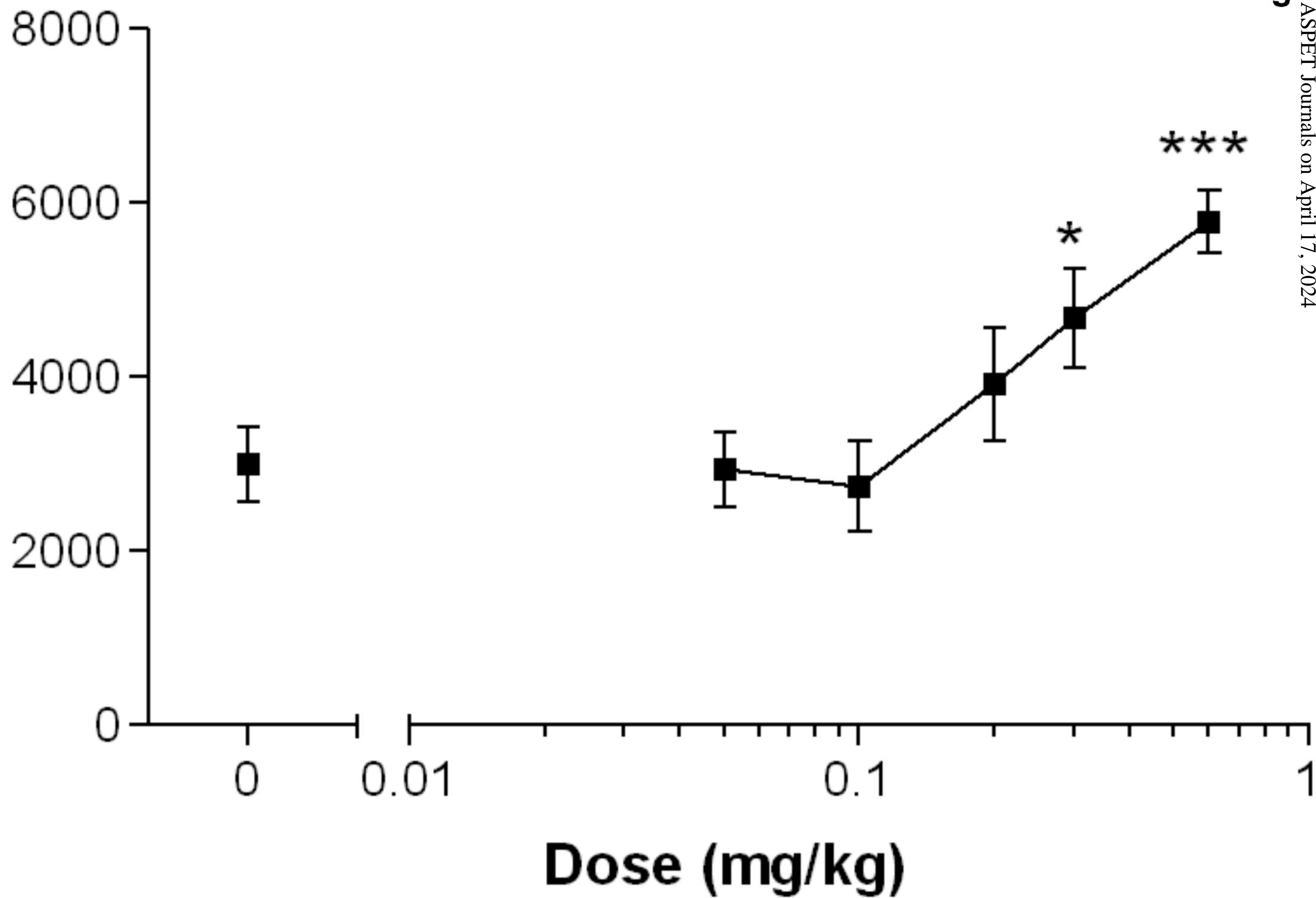

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Fig. 2

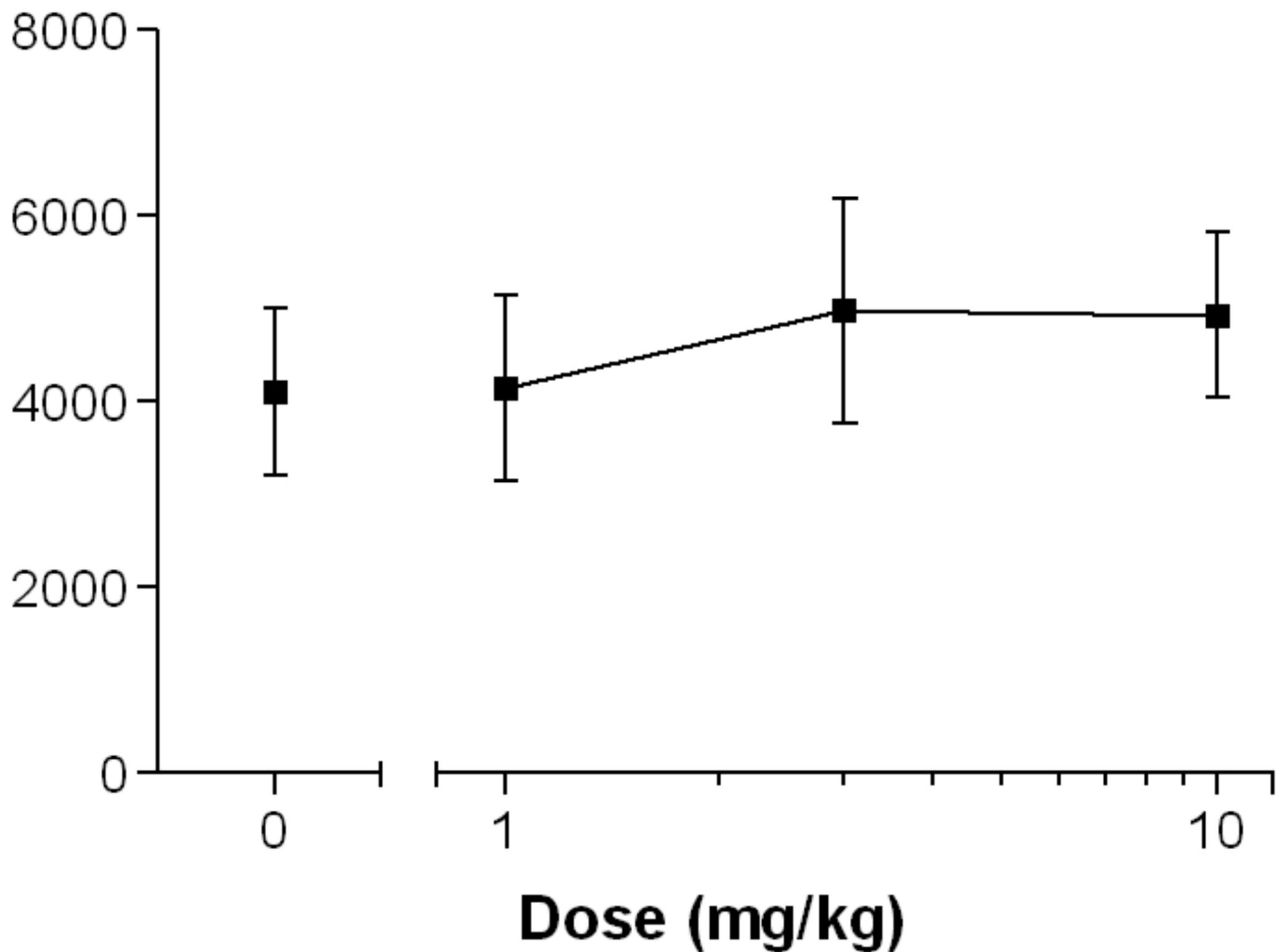

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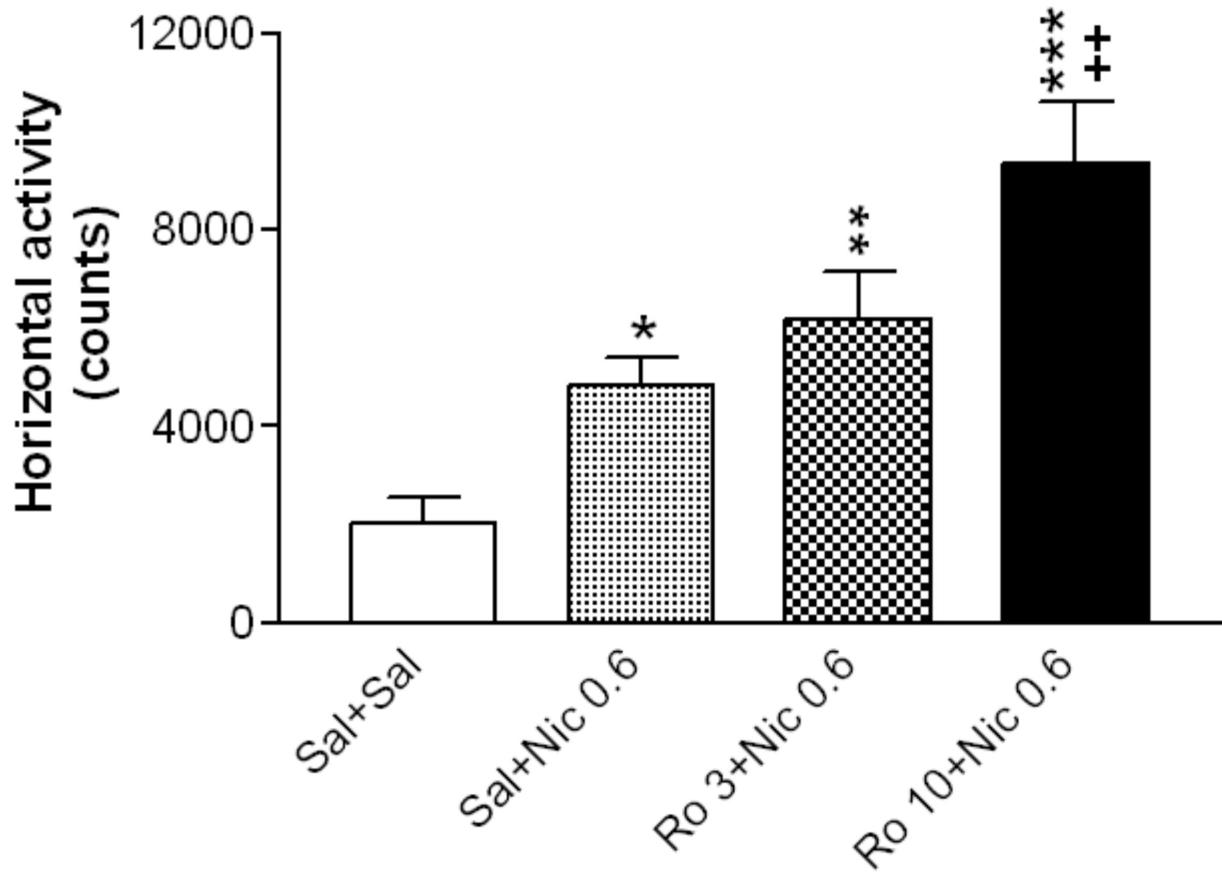
Fig.3



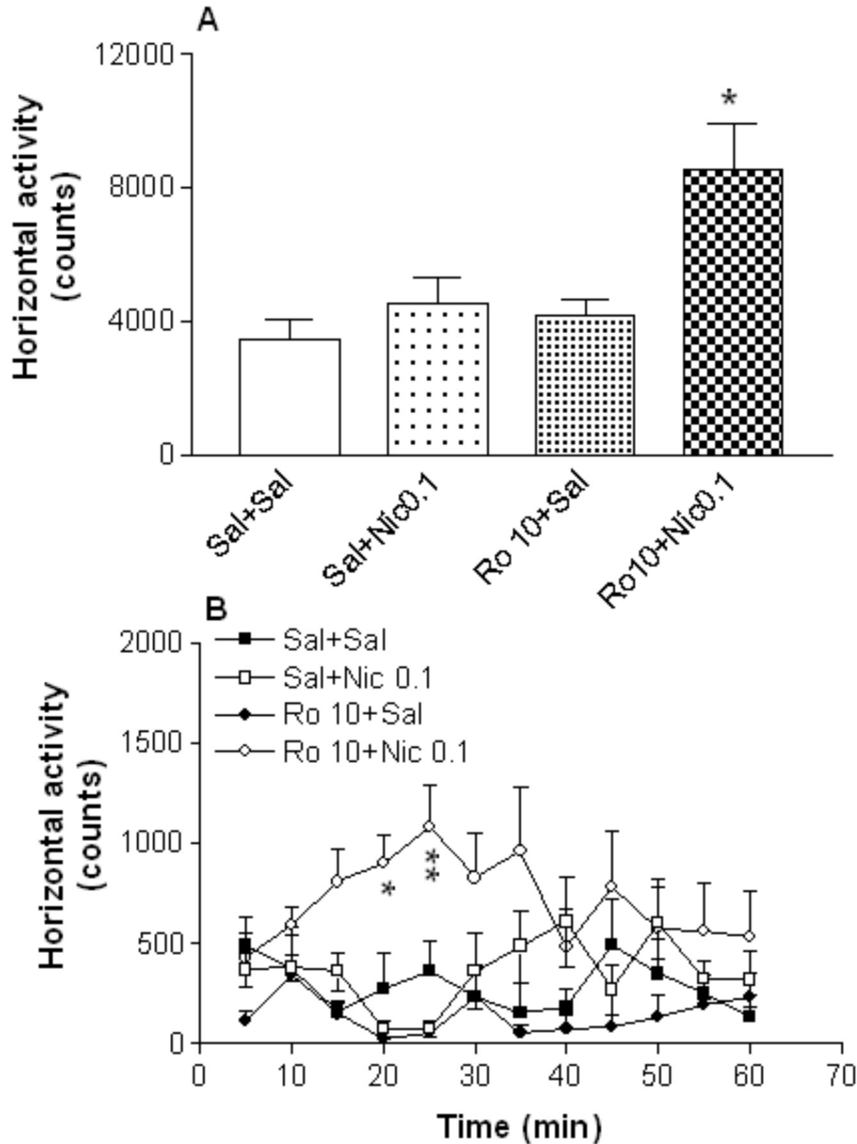


Fig. 4

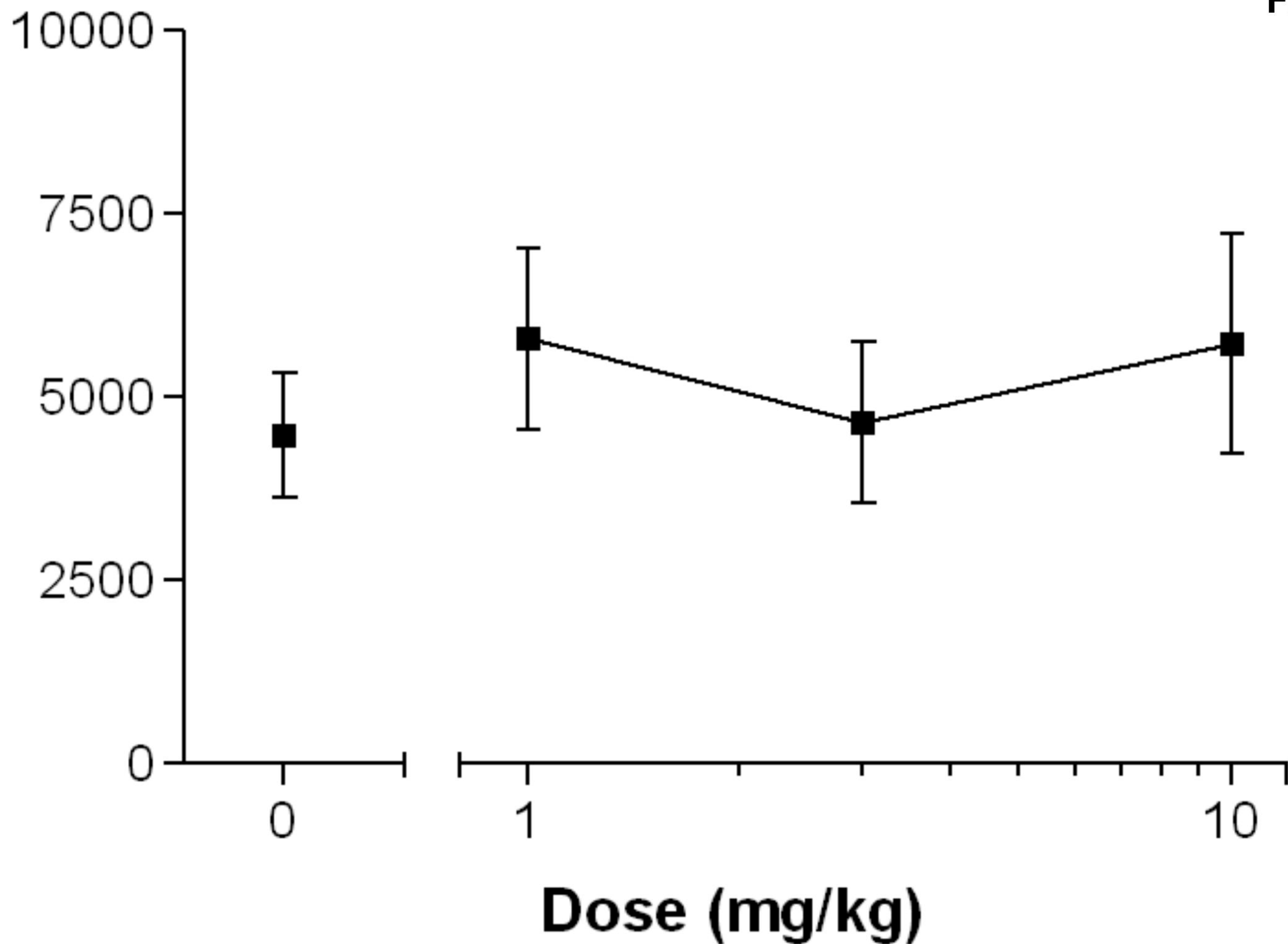


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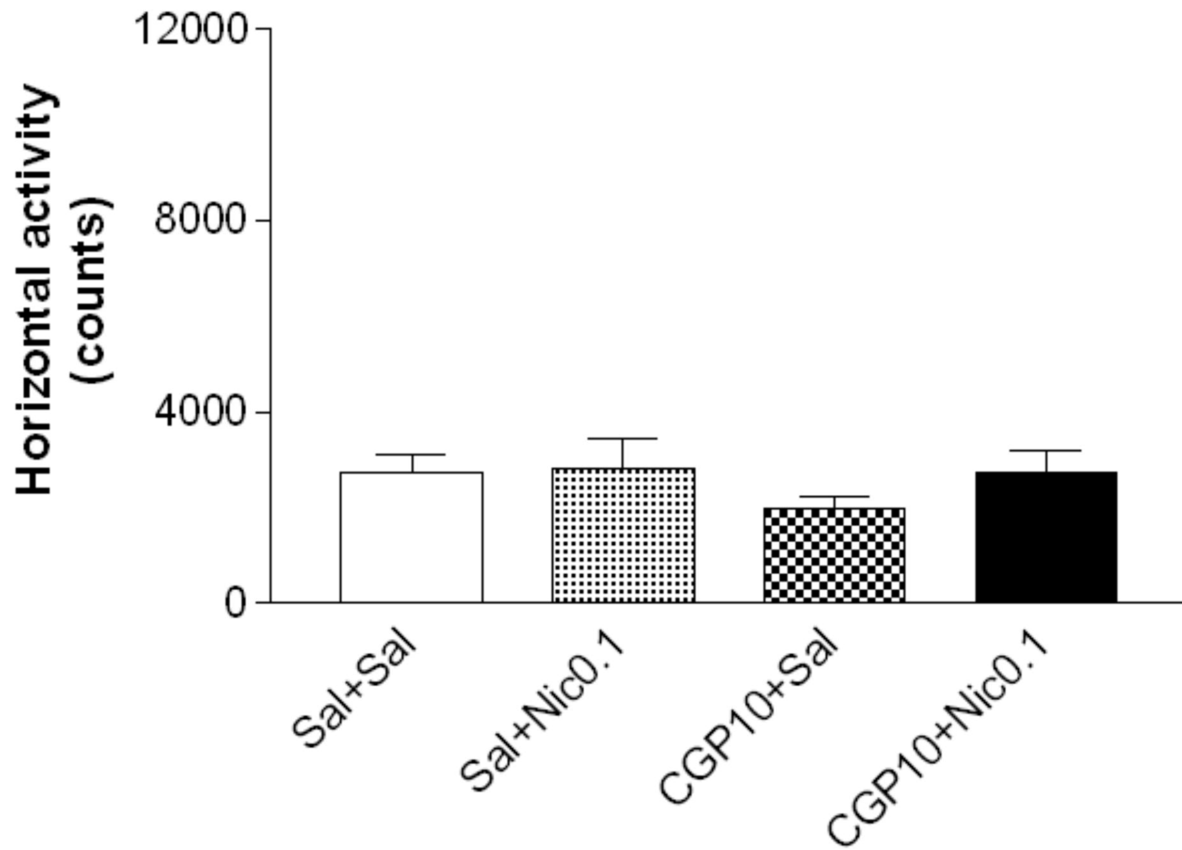
Fig. 6



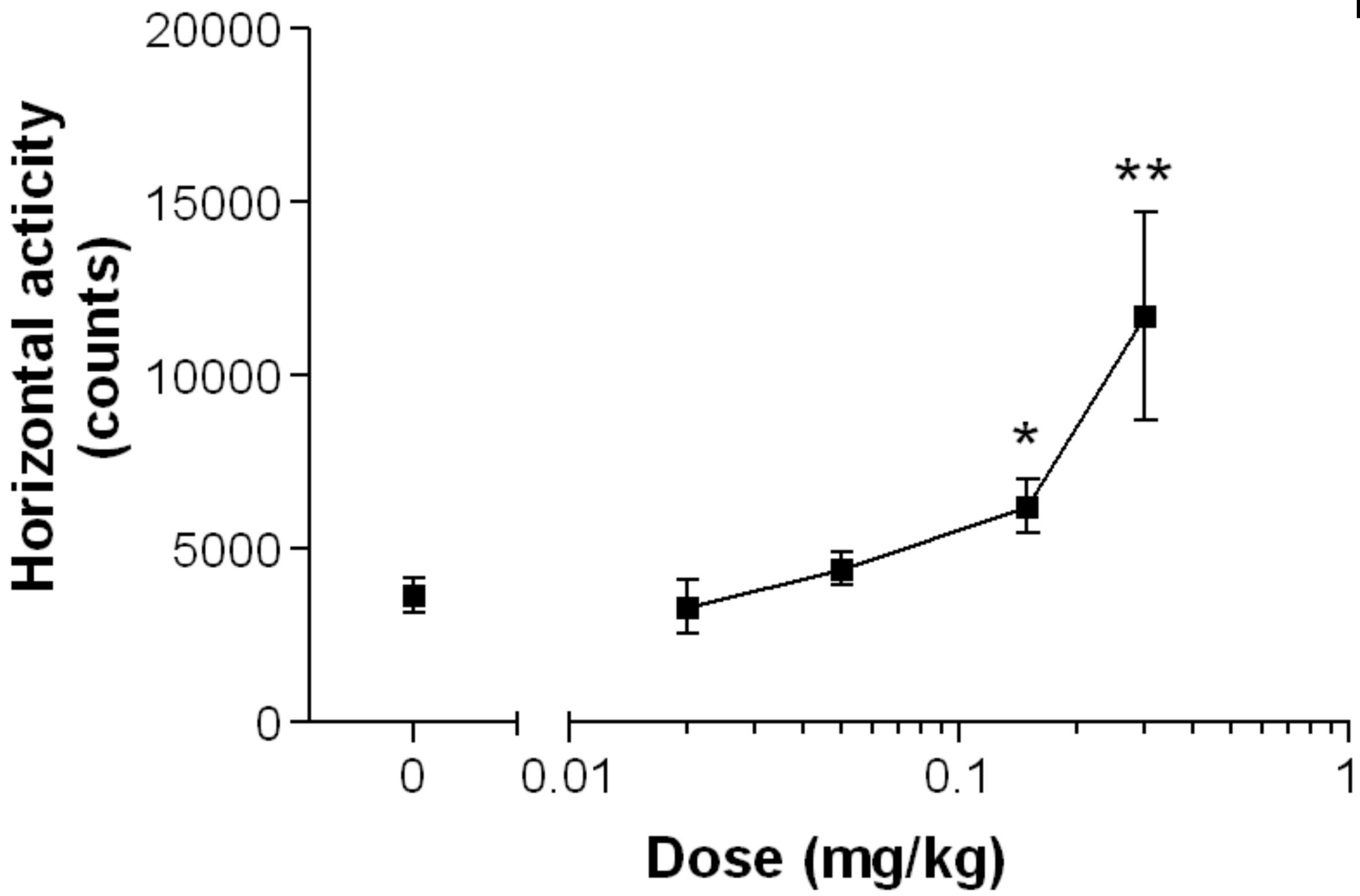


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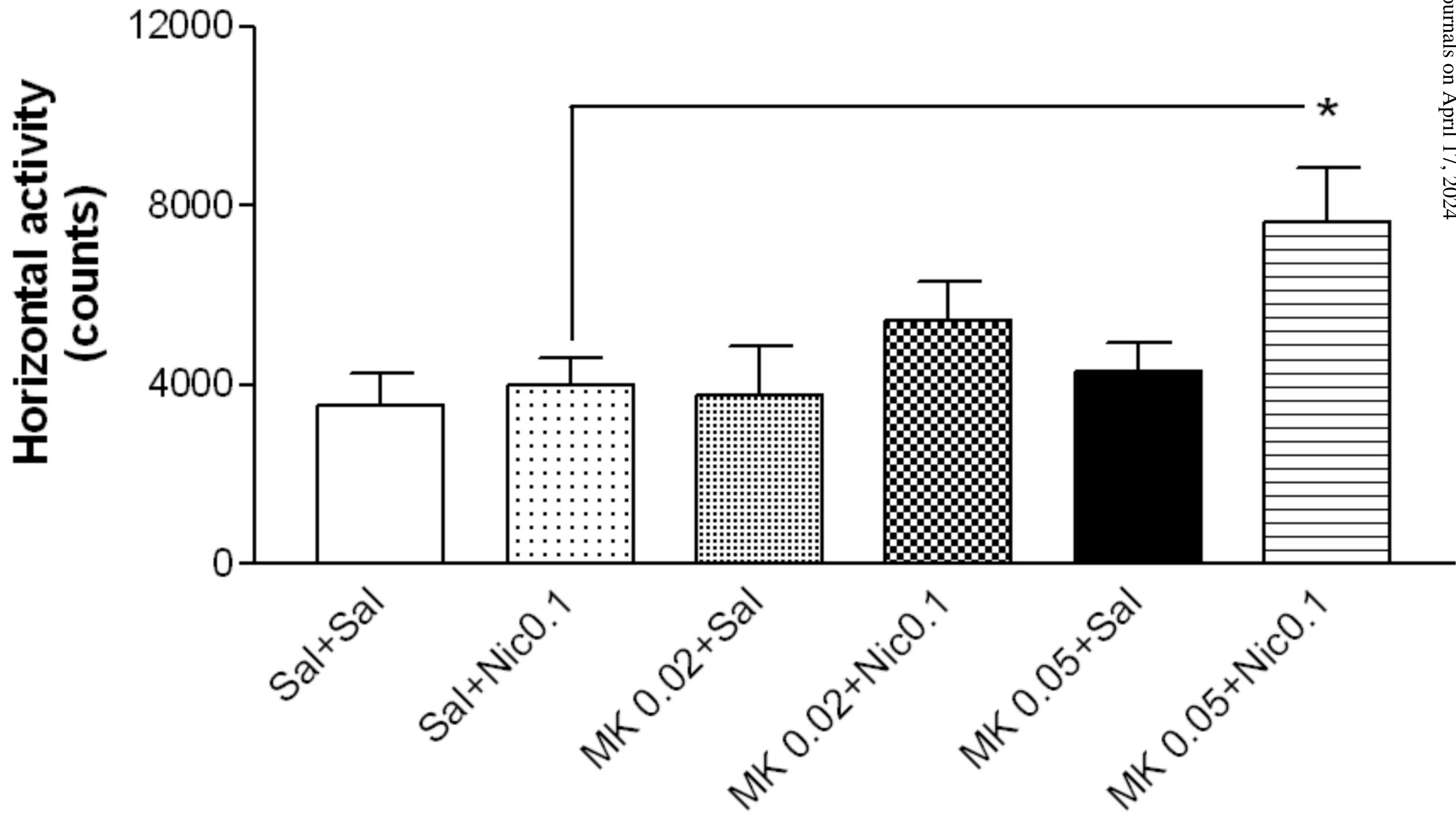
Fig. 8



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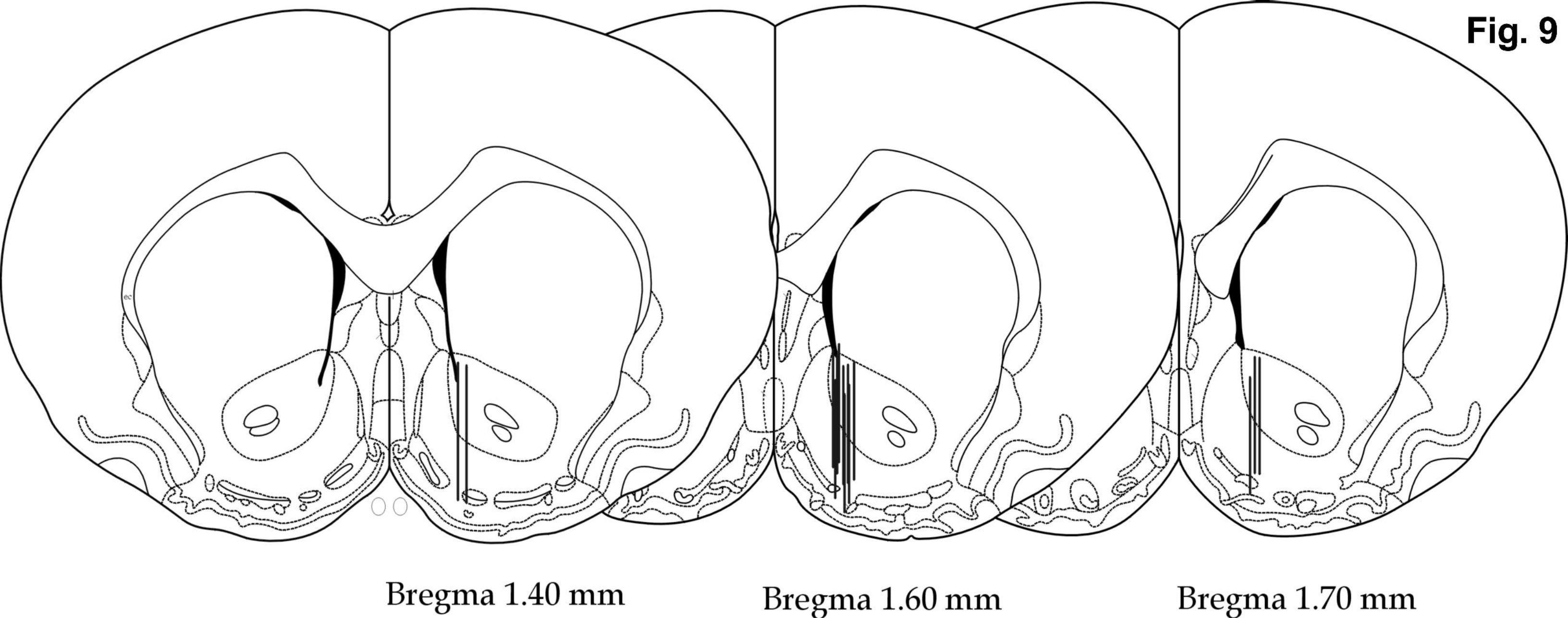
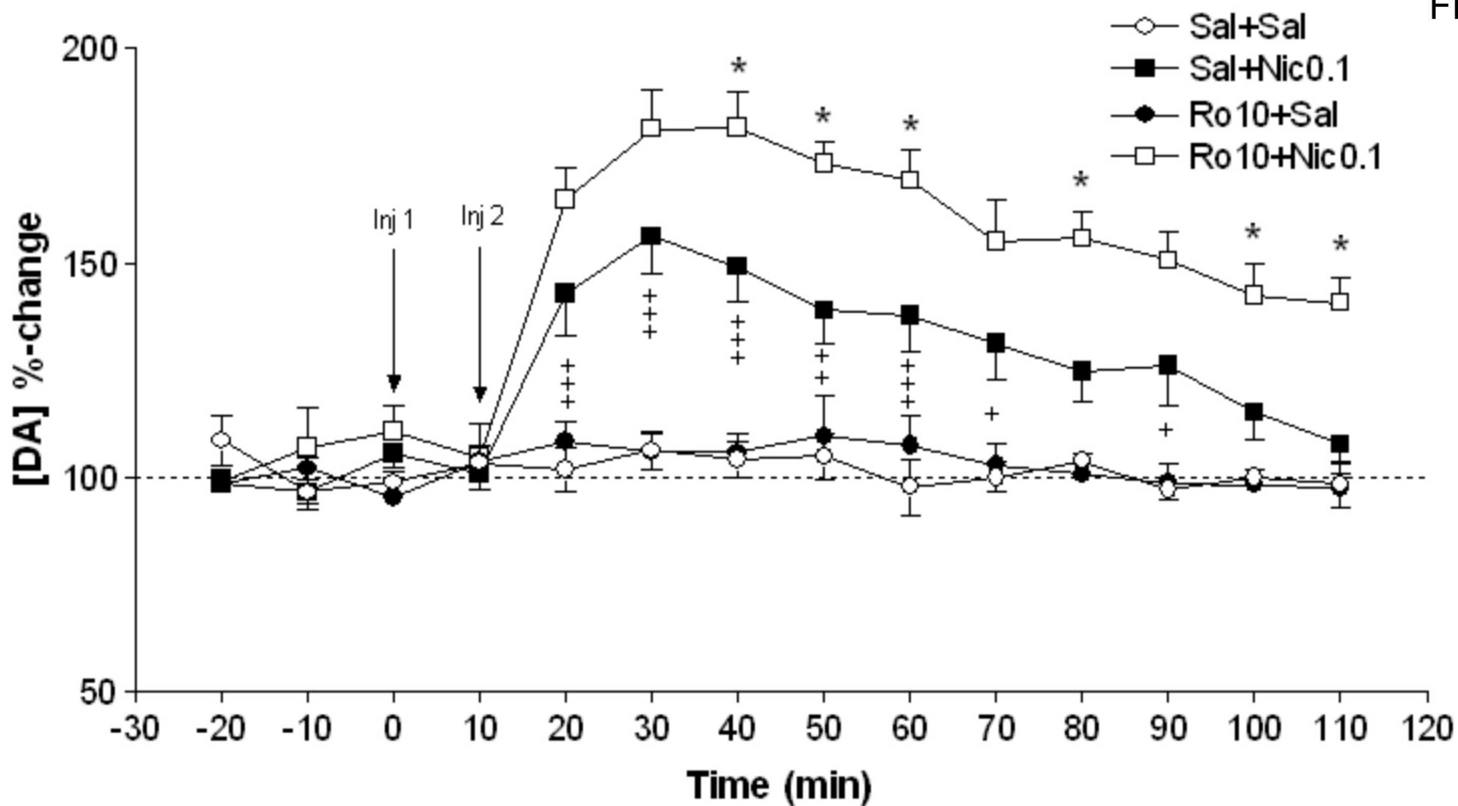
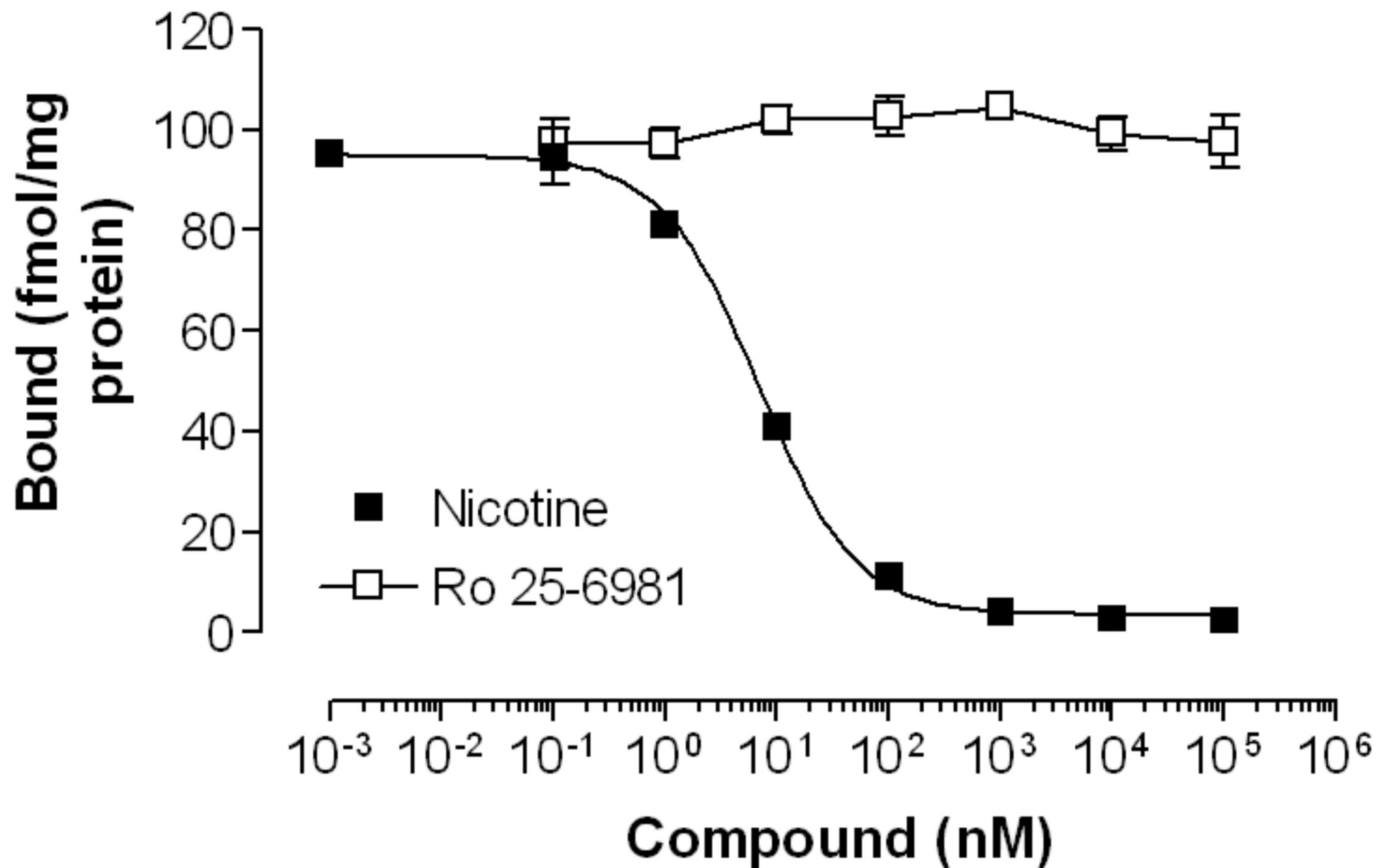


Fig. 9
ec
Bregma 1.40 mm
Bregma 1.60 mm
Bregma 1.70 mm

Fig. 10





Bound (fmol/mg protein)
120
100
80
60
40
20
0
Nicotine
Ro 25-6981
10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5} 10^{6}
Compound (nM)