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S33138, A PREFERENTIAL DOPAMINE D_3 *VERSUS* D_2 RECEPTOR ANTAGONIST AND POTENTIAL ANTIPSYCHOTIC AGENT. II. A NEUROCHEMICAL, ELECTROPHYSIOLOGICAL AND BEHAVIOURAL CHARACTERISATION *IN VIVO*

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Running Title: Dopamine D_3 receptors and schizophrenia

Abbreviations:

AR	Adrenoceptor
CT	Core temperature
5-CT	5-carboxytryptamine
DA	Dopamine
DOI	(1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane)
DRN	Dorsal raphe nucleus
DS	Discriminative stimulus
EPS	Extrapyramidal symptoms
FCX	Frontal cortex
5-HT	Serotonin
HTW	Head-twitches
IOC	Isles of Calleja
LC	Locus coeruleus
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NA	Noradrenaline
OH-DPAT	Dihydroxy-2-(di-n-propylamino)-tetralin
SNPC	Substantia nigra, pars compacta
VTA	Ventral tegmental area

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ABSTRACT

The novel benzopyranopyrrolidine, S33138 (N-[4-[2-[(3aS,9bR)-8-cyano-1,3a,4,9b-tetrahydro [1]benzopyrano[3,4-c]pyrrol-2(3H)-yl)-ethyl]phenylacetamide), is a preferential antagonist of cloned, human D_3 *vs* D_{2L} and D_{2S} receptors. In mice, S33138 more potently (0.04-2.5 mg/kg, i.p.) increased levels of mRNA encoding c-fos in D_3 receptor-rich Isles of Calleja and nucleus accumbens than in D_2 receptor-rich striatum. Further, chronic (3 weeks) administration of S33138 to rats more potently reduced the number of spontaneously-active dopaminergic neurones in the ventral tegmental area (0.16-10.0, p.o.) than in the substantia nigra (10.0). In primates treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, antiparkinson actions of the D_3/D_2 agonist, ropinirole, were *potentiated* by *low* doses of S33138 (0.01-0.16, p.o.) but diminished by a high dose (2.5). Consistent with antagonism of postsynaptic D_3/D_2 sites, S33138 attenuated hypothermia and yawns elicited by the D_3/D_2 agonist, 7-OH-DPAT, in rats, and it blocked (0.01-0.63, s.c.) discriminative properties of PD128,907. Suggesting antagonist properties at D_3/D_2 autoreceptors, S33138 prevented (0.16-2.5, s.c.) the inhibitory influence of PD128,907 upon dopamine release in frontal cortex, nucleus accumbens and striatum, and abolished (0.004-0.25, i.v.) its inhibition of ventral tegmental dopaminergic neurone firing. At higher doses, antagonist actions of S33138 (0.5-4.0, i.v.) at α_{2C}-adrenoceptors were revealed by an increased firing rate of adrenergic perikarya. Finally, antagonism of 5-HT_{2A} and 5-HT_7 receptors was shown by blockade of DOI-induced head-twitches (0.63-10.0, s.c.) and 5-carboxytryptamine-induced hypothermia (2.5-20.0, i.p.), respectively. In conclusion, S33138 displays modest antagonist properties at central α_{2C}-adrenoceptors, 5-HT_{2A} and 5-HT_7 receptors. Further, in line with its *in vitro* actions, it more potently blocks cerebral populations of D_3 *vs* D_2 receptors.

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INTRODUCTION

In the accompanying paper, the benzopyranopyrrolidine derivative, S33138, was shown to behave as a potent antagonist at human (h) dopamine D_3 receptors and, at higher concentrations, as an antagonist at hD_{2L} (long isoform) and hD_{2S} (short isoform) receptors. In addition, S33138 expressed modest antagonist properties at $h\alpha_{2C}$-adrenoceptors (ARs), serotonin $(5\text{-HT})_{2A}$ receptors and h5-HT_7 receptors, and weak antagonist actions at hD_1 receptors. S33138 possesses, thus, a cellular profile distinguishing it from previously documented antipsychotics. The purpose of the present studies was three-fold. *First*, employing a range of electrophysiological, neurochemical and behavioural procedures, to determine whether S33138 behaves as an antagonist at pre and postsynaptic populations of cerebral D_3 and D_2 receptors. *Second*, employing procedures in which the actions of selective D_3 *vs* D_2 antagonists have previously been documented (see citations below), to evaluate whether S33138 preferentially antagonises D_3 *vs* D_2 receptors *in vivo*. *Third*, to establish whether S33138 antagonises CNS populations of α_{2C}-ARs, 5-HT_{2A} receptors, 5-HT_7 receptors and D_1 receptors.

Activity of the cellular marker of neuronal activity, c-fos, is controlled by both D_3 and D_2 receptors. Selective D_3 *vs* D_2 receptor antagonists preferentially induce its gene expression in the Isles of Calleja (IOC) and nucleus accumbens (Kovacs et al., 2001; Ebert et al., 2005; Southam et al., 2007), structures enriched in D_3 receptors, as compared to the striatum where in D_2 receptors predominate (Landwehrmeyer et al., 1993; Joyce, 2001). Interest in the influence of S33138 upon c-fos is reinforced by studies indicating that the atypical antipsychotic, clozapine - but not haloperidol - also preferentially enhances c-fos expression in the IOC and nucleus accumbens (Vahid-Ansari and Robertson, 1992; Merchant et al., 1996; Guo et al., 1998; Carta and Gerfen, 1999; Kovacs et al., 2001). A further parallel between selective D_3 receptor antagonists and clozapine is that, upon chronic administration, both preferentially decrease the number of spontaneously-active dopaminergic neurones in the ventral tegmental area (VTA) *vs* the substantia nigra, pars compacta (SNPC) (Ashby and Wang, 1996, Ashby et al., 2000). This approach likewise suggests that blockade of D_3 receptors principally modulates the activity of D_3 receptor-rich limbic *vs* striatal structures.

D_3 autoreceptors are co-localized with D_{2S} receptors on dopaminergic perikarya and terminals (Stanwood et al., 2000; Usiello et al., 2000; Joyce, 2001; Joyce and Millan, 2005) where they play complementary roles in controlling the synthesis, release and clearance of dopamine (DA), as well as the electrical activity of dopaminergic neurones (Millan et al., 2000d; Usiello et al., 2000; Roberts et al., 2006; Sokoloff et al., 2006). Blockade of tonically-active, mesolimbic D_{2S} autoreceptors by haloperidol enhances DA release in the nucleus accumbens (Millan et al., 2000d), whereas their activation by the partial agonist, aripiprazole may, in moderating limbic DA release, contribute to its antipsychotic properties (Davies et al., 2004). Inasmuch as acute

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administration of selective D_3 receptor antagonists abrogates the *phasic* suppression of DA release and VTA firing by the preferential D_3 vs D_2 receptor agonist (op. cit.), PD128,907, we examined the influence of S33138 upon its actions.

Discriminative stimulus (DS) properties of dopaminergic agonists reflect recruitment of D_3 and/or D_2 autoreceptors (Cory-Slechta et al., 1996; Bristow et al., 1998; Millan et al., 2000b). By contrast, yawning involves postsynaptic D_2 and/or D_3 receptors on oxytocinergic neurones in the paraventricular nucleus of the hypothalamus (Argiolas and Melis, 1998; Chen et al., 1999; Millan et al., 2000a; Collins et al., 2005). As regards the hypothermic actions of dopaminergic agonists, integrated in the IOC, perifornical hypothalamus and other structures, the contribution of postsynaptic D_3 *vs* D_2 receptors remains controversial (Barik and Beaurepaire, 1998; Boulay et al., 1999; Millan et al., 2000a; Perachon et al., 2000; Chaperon et al., 2003). Interestingly, there is compelling evidence for a *contrasting* influence of postsynaptic D_3 *vs* D_2 sites upon motor function. Thus, blockade of mesolimbic and striatal D_2 sites disrupts motor behaviour, whereas the selective inactivation of D_3 receptors *enhances* motor behaviour (Boulay et al., 1999; Millan et al., 2000a; Joyce, 2001; Sokoloff et al., 2006). This opposing influence of D_3 *vs* D_2 receptors can be revealed in primates rendered parkinsonian by the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). In such akinetic subjects, improvement of motor performance by antiparkinson agents is potentiated and abrogated by selective antagonists at D_3 and D_2 receptors, respectively (Silverdale et al., 2004; Hill et al., 2006). In light of the differential implication of D_3 and D_2 sites in these behavioural procedures, they were also used to examine the actions of S33138.

Dopamine D_1 receptors in limbic and striatal regions control motor drive and coordination, as reflected in the induction of rotation by agonists in rats sustaining a unilateral lesion of the SNPC (Gulwadi et al., 2001; Gerfen et al., 2002). Thus, we examined the influence of S33138 upon rotation elicited by SKF81297. Despite the significance of α_{2C}-ARs to cognition and mood (Svensson, 2003), *in vivo* models of drug actions at α_{2C}-AR sites remain to be established. Nonetheless, α_{2C}-AR autoreceptors regulate the activity of adrenergic perikarya in the locus coeruleus (LC) (Arima et al., 1998; Millan et al., 2000d; Owesson et al., 2003), so the influence of S33138 upon its firing rate was determined in anesthetised rats. Finally, activation of 5-HT_{2A} receptors by the agonist, 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI), elicits head-twitches (HTW) in rats (Schreiber et al., 1995; Willins and Meltzer, 1997), while stimulation of 5-HT_7 receptors in guinea pigs evokes hypothermia (Hagan et al., 2000; Hedlund and Sutcliffe, 2004). Correspondingly, potential antagonist actions of S33138 at these sites were evaluated using these procedures.

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METHODS

Animals. Unless otherwise specified, *in vivo* studies employed male Wistar rats weighing 220-250 g (Iffa-Credo, L'Arbresle, France). Male CD1 mice weighing 22-25 g (Charles River, Saint-Aubin-les-Elbeuf, France), male C57/Bl6 mice weighing 20-25 g (for c-fos studies) and male Hartle guinea pigs weighing 200-250 g (Charles River, Saint-Aubin-les-Elbeuf, France) were also used. Subjects were maintained in sawdust-lined cages with unrestricted access to food and water. The laboratory temperature was held at 21 ± 1 °C and humidity was controlled at 60 ± 5 %. There was a 12 hr/12 hr light-dark cycle, with lights on from 7:30 to 19:30 h. Prior to experimentation, all animals were adapted for at least 1 week to laboratory conditions. All animals use procedures conformed to international European ethical standards (86/609-EEC) and the French National Committee (décret 87/848) for the care and use of laboratory animals. In primate studies, adult marmosets (*Callithrix jacchus*) were used. They were kept in controlled housing conditions with the temperature held at 26 ± 1 °C, 50 % relative humidity and a 12-h light/dark cycle (lights on from 7:30 AM to 19:30 PM). They had free access to food pellets, fresh fruit supplements and water. All primate experiments were carried under Home Office License PPL 40/01487 in accordance with UK legal requirements.

Drug testing. Unless otherwise specified, S33138 was examined by the subcutaneous route and full dose-response curves were generated. In the protocols of D_3 and D_2 receptor mediated activity, we have characterised the actions of selective D_3 (and D_2) receptor antagonists in previous studies (see Discussion). The equilibrated D_2/D_3 receptor antagonist, haloperidol, was employed herein as an internal reference ligand to "validate" this series of experiments: data are summarized in the Results section. For protocols of activity at D_1 receptors, α_{2C}-ARs, 5-HT_{2A} receptors and 5-HT_7 receptors, the selective antagonists, SCH23390, idazoxan, MDL100,907 and SB269,970, respectively, were similarly used as “internal” validators in parallel with S33138 (see Results).

Influence of S33138 upon cerebral levels of mRNA encoding c-fos. In mice, the influence of a single i.p. injection of S33138 upon levels of mRNA encoding c-fos was evaluated in cerebral structures differentially expressing D_3 *vs* D_2 receptors (IOC, nucleus accumbens and striatum) employing *in situ* hybridization as described in detail previously (Svenningsson et al., 1997). Briefly, mRNA levels encoding c-fos were quantified in coronal, cryostat brain sections using a [^{35}S]-labeled cRNA probe and standard hybridization and washing conditions. After washing, sections were dried and apposed to betaMAX hyperfilm for four weeks. Autoradiograms were quantified with a Microcomputer Imaging Device system using NIH Image 1.62 software. Slides were subsequently dipped in liquid photographic emulsion and counterstained in cresyl violet for cellular analyses of neurons labelled for c-fos.

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Influence of chronic S33138 upon spontaneously-active dopaminergic neurones in the VTA *vs* SNPC of anesthetized rats. The procedure used was essentially as previously described (Ashby et al., 2000). Male Sprague-Dawley rats (Taconic Farms, NY) (150-175 g at the beginning of the treatment) received one p.o. administration of vehicle or S33138 (0.16 to 10.0 mg/kg) or the "positive control", haloperidol (0.5 and 2.0 mg/kg), once a day for 21 consecutive days. All experiments were conducted 2 hours after the last administration on Day 21. Animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and mounted in a stereotaxic instrument. Single barrel microelectrodes (glass borosilicate, World Precision Instruments, Sarasota, FL) were used for recording the electrical activity of single dopaminergic neurones in the A10, VTA (AP: 3.0-3.5 mm; L: 0.5-1.0 mm and DV: 6.0-8.5 mm to the cortical surface) and the A9, SNPC (AP: 3.0-3.5 mm to lambda, L: 1.8-2.5 mm and DV: 6.0-8.5 mm). The number of spontaneously-active dopaminergic neurons in each region was determined across ten stereotaxic descents.

Influence of S33138 upon the actions of the D_3/D_2 receptor agonist, ropinirole, in parkinsonian primates. The procedure employed was essentially that described previously (Silverdale et al., 2004; Hill et al., 2006). Briefly, six adult marmosets were rendered parkinsonian by subcutaneous injection of 2 mg/kg of MPTP for 5 consecutive days. This produced a parkinsonian state that was stable after 18 weeks. Marmosets were then treated orally with 12.5 mg/kg of L-(3,4-dihydroxyphenyl)-alanine and 3.1 mg/kg benserazide (as Madopar® 62.5 mg dispersible, Roche, dissolved in apple juice) twice daily for 4 weeks. Thereafter, the studies with S33138 were performed. On the day of experiment, ropinirole plus vehicle, or ropinirole plus S33138, were administered orally in volumes of 5 ml/kg. In a separate experiment, S33138 or vehicle were administered in the absence of ropinirole. Immediately after treatment, the animals were transferred to an observation cage (60 cm x 55 cm x 75 cm). Locomotor activity was monitored for 2 consecutive hours using computer-based infrared activity monitors which provide a quantitative assessment of the total amount of movement in each 5-min time period throughout the experiment.

Influence of S33138 upon the hypothermia induced by the preferential D_3 *vs* D_2 receptor agonist, 7-OH-DPAT. Core temperature (CT) was determined in lightly-restrained rats by use of a rectal thermistoprobe as described previously (Millan et al., 2000a). Basal CT was measured, S33138, haloperidol or vehicle were administered s.c. and, 30 min later, 7-OH-DPAT (0.16 mg/kg, s.c) or vehicle were injected. Thirty min later, CT was re-evaluated and the difference (Δ) to pre-treatment values calculated.

Influence of S33138 upon the yawns elicited by 7-OH-DPAT. As previously described (Millan et al., 2000a), rats (120-140 g) were individually placed in plexiglass observation cages (11 x 26 x 30.5 cm) behind which mirrors were positioned to facilitate monitoring of behaviour.

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Thirty min after injection of S33138, haloperidol or vehicle, 7-OH-DPAT (0.04 mg/kg, s.c.) was administered and the number of yawns measured over a 30 min observation period.

Influence of S33138 upon DS properties of the preferential D_3 *vs* D_2 receptor agonist, PD128,907. Employing a procedure detailed in previous work (Millan et al., 2000b), rats were trained to discriminate PD128,907 (0.16 mg/kg, i.p.) from saline using a two-lever, Fixed-Ratio 10, food-reinforced procedure. Each 15-min daily session started 15 min following injection. The discrimination criterion was 10 consecutive sessions of correct responses (defined as a maximum of 13 responses on both reinforced and non-reinforced levels before the first reinforcement). Thereafter, tests were performed on Wednesdays and Fridays, and training sessions on the other days. Only rats responding appropriately on the two most recent training days were examined. In test sessions, responses on the selected lever (on which 10 responses were emitted first) were reinforced for the rest of the session. S33138 or haloperidol were injected s.c. 30 min prior to PD128,907. Data recorded during the test sessions were lever selection and response rate (the total number of presses on both levers).

Blockade by S33138 of the inhibitory influence of PD128,907 upon the electrical activity of dopaminergic cell bodies. The procedure used has been detailed previously (Millan et al., 2000c, 2004a). Following anaesthesia with chloral hydrate (400 mg/kg, i.p.) and femoral vein cannulation, rats were placed in a stereotaxic apparatus and a tungsten microelectrode was lowered by an electronic microdrive (Unimechanique, Epinay sur Seine, France) into the VTA (AP: -5.5 from bregma, L: 0.7, H: -7/-8.5 from dura) for recording of extracellular unit activity. Dopaminergic neurones were characterized by their distinctive waveforms and discharge patterns (Millan et al., 2000c). One cell was recorded in each animal. Following baseline recording (≥ 5 min), PD128,907 (0.01 mg/kg, i.v.) or vehicle were injected: 1 min later, S33138 (0.001-0.25 mg/kg, i.v., one dose per rat) or vehicle were injected and firing rate again recorded. In a separate experiment, the influence of cumulative (every 2-3 min) administration of S33138 (0.125-4.0 mg/kg, i.v.) *vs* vehicle was examined. At the end of the treatment, the prototypical dopaminergic agonist, apomorphine (0.031 mg/kg, i.v.), was injected and firing rate again recorded. Haloperidol was evaluated at a single dose of 0.031 mg/kg, i.v.. Drug effects were quantified over a 60 sec bin at the time of peak action. Spike2 V5.11 software (CED, Cambridge, England) was used for data acquisition and off-line analysis. Drug effects are expressed as percent change from baseline (pre-drug) firing rate (defined as 0 %).

Blockade by S33138 of the inhibitory influence of PD128,907 upon dialysate levels of dopamine in freely-moving rats. Extracellular levels of DA in single dialysate samples of the frontal cortex (FCX), nucleus accumbens and striatum were determined as previously (Millan et al., 2000c, 2004a). Briefly, guide cannulae were implanted under pentobarbital anaesthesia (60 mg/kg, i.p.) 1 week prior to experimentation and, on the test day, a cuprophane CMA/11

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probe (4 mm in length for the FCX and striatum, 2 mm for the nucleus accumbens and, in each case, 0.24 mm outer diameter) was lowered into position. Three basal samples of 20 min each were taken. Vehicle, S33138 or haloperidol were administered s.c. followed, 20 min later, by PD128,907 (0.16 mg/kg, s.c.) or vehicle and samples taken for another 3 hrs. DA levels were quantified by high performance liquid chromatography followed by amperometric detection. The assay limit of sensitivity was 0.1-0.2 pg/sample.

Influence of S33138 upon rotation elicited by the D_1 receptor agonist, SKF81297, in rats with a unilateral lesion of the SNPC. As previously described (Millan et al., 2000a, 2004b), rotation was measured in rats (350-400g at the time of the study) that had received a unilateral, 6-hydroxydopamine (8 μg/4 μl) lesion of the SNPC. Rotation was measured by use of a Rotacount 8 (Columbus Instruments, Columbus, OH) apparatus. Sessions were performed weekly employing an "ABACADA" design where "A" corresponds to injection of the selective D_1 agonist, SKF81297 (0.04 mg/kg, s.c.) (control sessions), and "B, C, and D" to test sessions with s.c. administration of S33138 or the selective D_1 receptor antagonist SCH23390, followed by SKF81297. In SKF81297-control sessions, vehicle was given 30 min before SKF81297 and rotation measured for 1 hr. In test sessions, S33138 or SCH23390 were given 30 min prior to SKF81297 and rotation likewise measured for 1 hr. Animals were, thus, their "own" controls.

Influence of S33138 upon the electrical activity of adrenergic as compared to serotonergic cell bodies. The basic procedure used was as described above for the VTA and documented previously (Millan et al., 2000c, 2004a). Thus, a tungsten microelectrode was lowered by an electronic microdrive into the LC (AP: -1.0 from zero, L: 1.0-1.2, H: -5.5/-6.0 from the sinus surface) of anesthetized rats. Adrenergic neurones were characterized by their distinctive waveforms and discharge patterns (Millan et al., 2000c; Millan, 2004a). One cell was recorded in each animal. Following baseline recording (≥ 5 min) and vehicle injection (0.5 ml/kg, i.v.), S33138 was administered in cumulative doses every 2-3 min. Subsequent to S33138 administration, a further injection was made of the α_2-AR autoreceptor agonist, clonidine (0.01 mg/kg, i.v.), followed by the α_2-AR antagonist, idazoxan (0.063 mg/kg, i.v.). In a separate group of subjects, the influence of idazoxan alone was also tested. In a further study, the influence of S33138 upon the activity of serotonergic neurones in the dorsal raphe nucleus (DRN) was examined. Coordinates were AP: -7.8 from bregma, L: 0, H: -5/-6.5 from the sinus surface. Cumulative administration of S33138 was followed by injection of the 5-HT_{1A} receptor agonist, 8-OH-DPAT (0.005 mg/kg, i.v.), followed by the selective 5-HT_{1A} antagonist, WAY100,635 (0.1 mg/kg, i.v.). In a separate experiment, WAY100,635 was injected followed by 8-OH-DPAT. Effects of S33138 were quantified over 60 sec bins at the time of peak action. Drug effects were expressed *vs* baseline (pre-drug) firing rate (defined as 0 %).

Influence of S33138 upon the induction of HTW by the 5-HT_{2A} receptor agonist, DOI, in rats. Using a procedure described previously (Schreiber et al., 1995), DOI (2.5 mg/kg, i.p.) was injected to rats placed in plexiglass observation cages (33.5 x 23.5 x 19 cm). Five min after DOI, the number of HTW made in 5 min was counted. Vehicle, S33138 or the selective 5-HT_{2A} receptor antagonist, MDL100,907, were given s.c. 30 min before DOI.

Influence of S33138 upon the induction of hypothermia by the 5-HT_7 receptor agonist, 5-carboxytryptamine (5-CT), in guinea pigs. Essentially as described elsewhere (Hagan et al., 2000), CT was determined in lightly-restrained male guinea pigs with a digital thermistoprobe placed into the ear for 30 seconds. Basal CT was measured, S33138, the selective 5-HT_7 receptor antagonist, SB269,970, or vehicle were administered i.p. and, 30 min later, 5-CT (3 mg/kg, i.p.) or vehicle were injected. Ninety min later, CT was re-evaluated and the difference (Δ) to pre-treatment values calculated.

Drug salts and sources. Haloperidol and S33138 were dissolved in sterile water plus a few drops of lactic acid, and the pH adjusted close to neutrality (pH ≥ 5.5) using NaOH. All other drugs were dissolved in sterile water. Doses are in terms of the base. Drug structures, sources and salts were as follows: MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) HCl was purchased from Sigma, Dorset, UK. Clonidine HCl, DOI ((±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane) HCl, 7-OH-DPAT ((+)-7-hydroxy-2-(di-n-propylamino)-tetralin) HCl, 8-OH-DPAT ((±)-8-hydroxy-2-(di-n-propylamino)-tetralin) HBr, PD128,907 ((+)-(4aR,10bR)-3,4,4a,10b-tetrahydro-4-propyl-2H, 5H-[1]benzopyrano-[4,3-*b*]-1,4-oxazin-9-ol) HCl and SCH23390 (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine) HCl were obtained from Research Biochemicals International (Natick, MA). Apomorphine, idazoxan, haloperidol, SKF81297 ((±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine) HBr and benserazide HCl were acquired from Sigma Chimie (St Quentin-Fallavier, France). S33138 (N-[4-[2-[(3aS,9bR)-8-cyano-1,3a,4,9b-tetrahydro[1]benzopyrano[3,4-c]pyrrol-2(3H)-yl)-ethyl]phenyl-acetamide) HCl, 5-carboxy-tryptamine, maleate and MDL100,907 (R(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol) were synthesised by G. Lavielle (Servier). Ropinirole, SB269,970 (R)-1-{2-[1-(3-hydroxy benzensulfonyl) pyrrolidin-2-yl] ethyl}-4-methylpiperidine) HCl and WAY100,635 ((N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-(2-pyridinyl)cyclo-hexanecarboxamide) fumarate were synthetized by J.-L. Péglion (Servier).

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RESULTS

Influence of S33138 upon c-fos gene expression in cerebral structures differentially expressing D_3 as compared to D_2 receptors (Figs. 1 and 2). S33138 dose-dependently (0.04-2.5 mg/kg, i.p.) elevated levels of mRNA encoding c-fos in the IOC of mice, a limbic region containing D_3 but not D_2 receptors. S33138 also significantly elevated c-fos gene expression in the nucleus accumbens, a region enriched in D_3 receptors. Conversely, only a "high" dose of S33138 significantly elevated levels of mRNA encoding c-fos in the striatum, a D_2 receptor-rich structure possessing a few D_3 receptors. Haloperidol showed pronounced effects in all structures with the greatest increase in the striatum. Expressed relative to vehicle (100 %): IOC, vehicle = 100.0 ± 19.0; haloperidol, 0.5 mg/kg, 177.8 ± 49.2 and haloperidol, 2.0 mg/kg, 175.9 ± 19.0, $F(2,22) = 3.3$, $P < 0.05$. Nucleus accumbens, vehicle = 100.0 ± 10.0; haloperidol, 0.5 mg/kg, 189.8 ± 36.5 and haloperidol 2.0 mg/kg, 189.7 ± 25.1, $F(2,19) = 5.9$, $P < 0.05$ and striatum, vehicle = 100.0 ± 7.0; haloperidol, 0.5 mg/kg, 193.9 ± 21.5 and haloperidol, 2.0 mg/kg, 340.1 ± 53.3, $F(2,18) = 15.5$, $P < 0.001$.

Influence of chronic administration of S33138 on spontaneously-active dopaminergic neurones in anesthetized rats (Fig. 3). Following chronic (3 week) daily p.o. administration of vehicle, 1.21 ± 0.13 active neurons were identified in the VTA. Chronic administration of S33138 (0.16-10.0 mg/kg, p.o.) potently and dose-dependently reduced the number of active neurons. In contrast, S33138 decreased spontaneously-active neurons in the SNPC only at the highest dose of 10.0 mg/kg. Moreover, the maximal reduction in number of spontaneously-active neurons (- 58 %) was less than for the VTA (- 83%). Haloperidol (0.5 mg/kg, p.o.) produced a significant ($P < 0.01$) reduction in the number of active neurons in the VTA (- 66 + 6.6 %) and in the SNPC (- 62 ± 6.2 %) as compared to vehicle (0 %).

Influence of S33138 upon actions of the D_3/D_2 receptor agonist, ropinirole, in parkinsonian primates (Fig. 4). In marmosets exposed to MPTP, ropinirole (0.63 mg/kg, p.o.) increased motor activity as compared to vehicle: 3091 ± 556 *vs* 235 ± 132 counts, respectively ($P < 0.01$ in a two-tailed t-test). Over a low dose range, S33138 (0.0025-0.16 mg/kg, p.o.) dose-dependently and significantly enhanced this facilitatory influence of ropinirole. Conversely, a higher dose of S33138 (2.5 mg/kg) reduced the action of ropinirole. S33138 (0.16 mg/kg) did not significantly modify motor behavior when given alone (Fig. 4 and not shown).

Antagonism by S33138 of the hypothermia elicited by the preferential D_3 *vs* D_2 receptor agonist, 7-OH-DPAT (Fig. 5A). 7-OH-DPAT (0.16 mg/kg, s.c.) elicited a pronounced reduction in CT in rats. This hypothermia was attenuated by S33138 (0.04-2.5 mg/kg, s.c.) which did not affect CT alone. Haloperidol likewise blocked 7-OH-DPAT-induced hypothermia: vehicle + 7-OH-DPAT = -1.44 ± 0.15° C; haloperidol (0.02 mg/kg, s.c.) + 7-OH-DPAT = -0.65 ± 0.12 °C; haloperidol (0.04) + 7-OH-DPAT = 0.07 ± 0.15 °C and haloperidol

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(0.16) + 7-OH-DPAT = 0.57 ± 0.10 °C. N = 6-8 per dose, F (3,30) = 31.5, P < 0.01. Doses of 0.02, 0.04 and 0.16, significantly different from vehicle in Dunnett's test. Haloperidol did not affect CT alone (not shown).

Antagonism by S33138 of yawns elicited by 7-OH-DPAT (Fig. 5B). 7-OH-DPAT (0.04 mg/kg, s.c.) elicited yawning in rats. This response was blocked by S33138 (0.63-10.0 mg/kg, s.c.) which alone did not elicit yawns (not shown). Haloperidol also blocked yawns elicited by 7-OH-DPAT: vehicle + 7-OH-DPAT = 11.2 ± 1.1; haloperidol (0.01 mg/kg, s.c.) + 7-OH-DPAT = 9.4 ± 0.9; haloperidol (0.04) + 7-OH-DPAT = 6.1 ± 1.0 and haloperidol (0.16) + 7-OH-DPAT = 1.4 ± 0.7. N = 7-8 per dose, F (3,30) = 18.6, P < 0.01. Doses of 0.04 and 0.16, significantly different from vehicle (Dunnett's test). Haloperidol did not elicit yawns alone (not shown).

Antagonism by S33138 of the discriminative stimulus properties of the preferential D_3 *vs* D_2 receptor agonist, PD128,907 (Fig. 5C). PD128,907 (0.16 mg/kg, i.p.) elicited a stable DS in rats. S33138 (0.0025-0.63 mg/kg, s.c.) blocked its DS properties with an Effective $Dose_{50}$ (95 % confidence limits) of 0.02 (0.01-0.04) mg/kg, s.c.. S33138 did not significantly decrease response rates: for the dose of 0.63 mg/kg, + 23 ± 18 % *vs* previous control session (0 %). Haloperidol (0.0025-0.04 mg/kg, s.c.) also attenuated the PD128,907 cue, exerting a maximal 50 % inhibition at 0.04 mg/kg (N = 7). At this dose, there was a pronounced decrease (-75 ± 32 %, P < 0.01, paired t-test) in the response rate. At a higher dose of haloperidol (0.08 mg/kg), rats were unable to select a lever owing to motor perturbation.

Antagonism by S33138 of the inhibitory influence of PD128,907 upon the electrical activity of dopaminergic cell bodies (Fig. 6). The firing rate (baseline, 4.0 ± 0.3 Hz) of dopaminergic perikarya in the VTA was markedly reduced by PD128,907 (0.01 mg/kg, i.v.). S33138 (one dose per subject, 0.001-0.25 mg/kg, i.v.) blocked this action of PD128,907 with a minimally-effective dose of 0.004 mg/kg, i.v., and without itself affecting firing rate. Haloperidol (0.031 mg/kg, i.v.) likewise reversed the action of PD128,907 and yielded an "overshoot" in firing rate relative to baseline values (0 %): vehicle/vehicle, + 0.1 ± 2.7 %; PD128,907/vehicle, -99.9 ± 0.1 %; vehicle/haloperidol, + 46.1 ± 25.6 % and PD128,907/haloperidol + 58.7 ± 36.5 %, F (3,18) = 10.3, P < 0.01. In a separate experiment, cumulatively administered over a higher range of doses (0.125 - 4.0 mg/kg, i.v.), S33138 slightly increased firing rate with a maximal effect of + 29 % (1.0 mg/kg). Apomorphine (0.031 mg/kg, i.v.) *failed* to inhibit dopaminergic neurons (+ 1.7 ± 5.5 %) following administration of S33138 as compared to vehicle (- 98.2 ± 0.4 %): P < 0.01 (two-tailed t test). Following haloperidol, apomorphine was likewise ineffective (not shown).

Antagonism by S33138 of the suppressive influence of PD128,907 upon DA levels in the FCX, nucleus accumbens and striatum (Fig. 7). PD128,907 (0.16 mg/kg, s.c.) decreased dialysis levels of DA in the FCX of freely-moving rats, an action dose-dependently prevented

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by S33138 (0.16-2.5 mg/kg, s.c.) which did not influence DA alone. Haloperidol (0.63 mg/kg, s.c.) also blocked the action of PD128,907 and itself increased levels of DA. Relative to basal values (100 %), "area under the curve" analysis as follows: vehicle/vehicle, 98.1 ± 1.8 %; vehicle/PD128,907 (0.16), 82.2 ± 3.1 %; haloperidol/vehicle, 127.6 ± 2.9 % and haloperidol/PD128,907, 132.2 ± 5.9 %. N = 5-9 per group. Influence of PD128,907, $F(1,16) = 10.0$, $P < 0.01$; influence of haloperidol, $F(1,15) = 25.6$, $P < 0.01$ and interaction, $F(1,12) = 19.0$, $P < 0.01$. S33138 likewise abolished the influence of PD128,907 (0.16 mg/kg, s.c.) on levels of DA in the nucleus accumbens and the striatum (Fig 7C and D). Haloperidol (0.63 mg/kg, s.c.) also blocked the actions of PD128,907 in nucleus accumbens and striatum, and alone increased levels of DA. Nucleus accumbens: vehicle/vehicle, 93.7 ± 1.9 %; vehicle/PD128,907, 75.0 ± 3.2 %; haloperidol/vehicle, 153.1 ± 3.2 % and haloperidol/PD128,907, 154.8 ± 8.2 %. N = 5-8 per group. Influence of PD128,907, $F(1,13) = 6.9$, $P < 0.05$, influence of haloperidol, $F(1,12) = 55.3$, $P < 0.01$ and interaction, $F(1,11) = 18.7$, $P < 0.01$. Striatum: vehicle/vehicle, 96.5 ± 1.7 %; vehicle/PD128,907, 67.2 ± 2.7 %; haloperidol/vehicle, 179.0 ± 5.6 % and haloperidol/PD128,907, 205.7 ± 4.0 %. N = 5-8 per group. Influence of PD128,907, $F(1,14) = 20.1$, $P < 0.01$; influence of haloperidol, $F(1,13) = 25.6$, $P < 0.01$ and interaction, $F(1,11) = 305.0$, $P < 0.01$.

Lack of inhibition by S33138 of rotation elicited by the D_1 receptor agonist, SKF81297, in rats with a unilateral lesion of the SNPC. SKF81297 (0.04 mg/kg, s.c.) elicited marked contralateral rotation: 595.7 ± 74.3 *vs* vehicle, 10.1 ± 3.0 contralateral turns ($P < 0.01$ in an unpaired Student's t-test). This action of SKF81297 was not significantly affected (Matched paired test) by S33138 (2.5-40.0 mg/kg, s.c.). Vehicle + SKF81297, 609 ± 81; S33138 (0.16 mg/kg, s.c.) + SKF81297, 602 ± 108 and S33138 (10.0) + SKF81297, 760 ± 57, N = 5-8 per dose. In contrast, the potent (rat D_1 receptors, pK_i, 8.9) D_1 receptor antagonist, SCH23390, blocked induction of rotation by SKF81297. Vehicle + SKF81297, 580 ± 92; SCH23390 (0.0025) + SKF81297, 484 ± 64; SCH23390 (0.01) + SKF81297, 311 ± 66, SCH23390 (0.02) + SKF81297, 243 ± 77 and SCH23390 (0.04) + SKF81297, 56 ± 34. N = 5-6 per dose. $F(4,28) = 5.2$, $P < 0.01$. Doses of 0.02 and 0.04, significantly different from vehicle (Matched pairs tests).

Influence of S33138 upon the electrical activity of adrenergic and serotonergic cell bodies in anesthetized rats (Fig. 8A). S33138 dose-dependently (0.125-4.0 mg/kg, i.v.) increased the activity of LC-localized adrenergic neurons (baseline firing rate, 1.1 ± 0.2 Hz). Relative to basal values (0 %), S33138 exerted a maximal effect of + 177 ± 7 % at 4.0 mg/kg. Following S33138, the α_2-AR agonist, clonidine (0.01 mg/kg, i.v.), *still* inhibited firing (-100.0 ± 0.0 %). This effect was reversed by the α_2-AR (rat α_2-adrenoceptors, pK_i, 8.5) antagonist, idazoxan (0.063 mg/kg, i.v.), which yielded an "overshoot" of + 81 ± 16.3 %. Alone, idazoxan (0.063 mg/kg, i.v, N = 5) significantly (+ 142.0 ± 13.9 %, $P < 0.01$) increased the firing rate relative to vehicle (N = 5), (- 2.2 ± 1.3 %). In contrast to the LC, S33138 (0.125-4.0 mg/kg, i.v.) did not

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affect the electrical activity of DRN-localized serotonergic neurons (baseline firing rate, 1.2 ± 0.2 Hz). Following S33138, the 5-HT_{1A} receptor agonist, 8-OH-DPAT (0.005 mg/kg, i.v.), still abolished the activity of serotonergic perikarya (- 97.6 ± 2.4 %). This effect was reversed by the 5-HT_{1A} receptor antagonist, WAY100,635 (0.1 mg/kg, i.v.: + 29.0 ± 12.8 %, ($P < 0.01$).

Antagonism by S33138 of the induction of head-twitches in rats by the 5-HT_{2A} receptor agonist, DOI (Fig. 8B). DOI elicited HTW in rats. S33138, which did not itself elicit HTW (not shown), dose-dependently (0.63-10.0 mg/kg, s.c.) abrogated their induction. The potent (rat 5-HT_{2A} receptors, pK_i, 9.1) 5-HT_{2A} receptor antagonist, MDL100,907, also abolished induction of HTW: vehicle + DOI, 6.1 ± 0.7; MDL100,907 (0.0025 mg/kg, s.c.) + DOI, 5.2 ± 0.5; MDL100,907 (0.005) + DOI, 3.0 ± 1.1; MDL100,907 (0.01) + DOI, 0.8 ± 0.5 and MDL100,907 (0.04) + DOI, 0.0 ± 0.0. N = 5-8 per value, $F(4,31) = 11.0$, $P < 0.01$. Doses of 0.01 and 0.04, significantly different ($P < 0.05$) from vehicle in Dunnett's test. MDL100,907 itself did not elicit HTW (not shown).

Antagonism by S33138 of the induction of hypothermia by the "5-HT_7" receptor agonist, 5-carboxytryptamine, in guinea pigs (Fig. 8C). 5-carboxytryptamine (3.0 mg/kg, i.p.) elicited hypothermia in guinea pigs, an effect dose-dependently attenuated by S33138 (2.5-20.0 mg/kg, i.p.). S33138 did not modify CT alone (not shown). By analogy, the potent (rat 5-HT_7 receptors, pK_i, 8.8) 5-HT_7 receptor antagonist, SB269,970, also inhibited induction of hypothermia: Vehicle + 5-CT, - 1.81 ± 0.30° C; SB269,970 (0.63 mg/kg, i.p.) + 5-CT, - 1.56 ± 0.22 °C, SB269,970 (2.5) + 5-CT, - 0.50 ± 0.36 °C and SB269,970 (10.0) + 5-CT, - 0.22 ± 0.23 °C. N = 5-8 per value, $F(3,25) = 4.4$, $P < 0.01$. Doses of 2.5 and 10.0, significantly different ($P < 0.05$) from vehicle in Dunnett's test. SB269,970 did not affect CT alone (not shown).

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DISCUSSION

Selective induction of c-fos by S33138 in limbic structures. The preferential induction of c-fos by S33138 in D_3 receptor-rich limbic *vs* striatal regions (Landwehrmeyer et al., 1993; Joyce, 2001; Sokoloff et al., 2006) correlates well with its higher affinity for D_3 over D_2 sites. Further, this finding parallels similar actions of selective antagonists at D_3 *vs* D_2 receptors (Kovacs et al., 2001; Southam et al., 2007; Svenningsson P, et al., unpub. obs.). Interestingly, functional "MRI" imaging studies also revealed greater activation of neurones in limbic *vs* striatal structures by selective blockade of D_3 receptors (Schwarz et al., 2004). Preferential limbic *vs* striatal induction of c-fos has been related to a high margin between doses controlling positive symptoms *vs* those eliciting extrapyramidal symptoms (EPS) and a similar pattern of effects to S33138 is seen with atypical antipsychotics (Robertson et al., 1994; Guo et al., 1998). However, it is unclear whether D_3 receptors are involved in their effects. Thus, preferential D_3 *vs* D_2 receptor agonists blunted limbic induction of c-fos by clozapine in rats (Guo et al., 1998; Vahid-Ansari and Robertson, 1996). However, mice genetically deprived of D_3 receptors revealed no attenuation in the influence of clozapine which may engage cells *different* to D_3 antagonists (Merchant et al., 1996; Carta and Gerfen 1999; Kovacs et al., 2001).

Inhibition of spontaneously-active VTA neurones by long-term administration of S33138. By analogy to clozapine and olanzapine (Stockton and Rasmussen, 1996; Grace et al., 1997; Ashby and Wang, 2000), and in contrast to haloperidol, chronic administration of S33138 *selectively* decreased the number of spontaneously-active VTA *vs* SNPC dopaminergic neurones. Supporting a role of D_3 receptor blockade, the action of S33138 was expressed at low doses similar to those inducing c-fos in limbic structures. Further, a selective reduction in spontaneously-active VTA *vs* SNPC neurones was likewise seen employing highly-selective D_3 antagonists (Ashby et al., 2000; Macdonald et al., 2003). Chronic administration of haloperidol may decrease the number of spontaneously-active VTA *and* SNPC neurones by "depolarisation" blockade since manipulations that hyperpolarise neurones, such as apomorphine administration, reverse its effects (Grace et al., 1997). However, the influence of chronic SB-277011, a selective D_3 receptor antagonist, upon VTA neurones, was not blocked by apomorphine (Ashby et al., 2000). Accordingly, depolarization blockade is probably not involved in long-term effects of S33138. Rather, the reduction in spontaneously-active VTA neurones produced by S33138 and selective D_3 receptor antagonists may reflect feedback actions initiated in structures like the nucleus accumbens (Grace et al., 1997; Ashby et al, 2000).

Dose-dependent facilitation and attenuation by S33138 of the antiparkinson properties of ropinirole. MPTP selectively lesions nigrostriatal dopaminergic projections, provoking a parkinsonian-like syndrome alleviated by D_2/D_3 receptor agonists (Pearce et al., 1998; Millan et al., 2004b; Silverdale et al., 2004). Blockade of D_2 receptors by a "high" dose of S33138 likely

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underlies its abrogation of the motor-relief afforded by ropinirole. By contrast, mirroring the facilitatory influence of selective D_3 receptor antagonists (Silverdale et al., 2004; Hill et al., 2006), low doses of S33138 *potentiated* the actions of ropinirole. These findings accord well with the notion that D_2 receptors mediate the beneficial actions of antiparkinson agents, while D_3 receptor *blockade* enhances their efficacy and reduces dyskinesias (Bezard et al., 2003; Millan et al., 2004b, c; Silverdale et al., 2004; Hill et al., 2006; Sokoloff et al., 2006).

Antagonist properties of S33138 in behavioural models. Though D_2 receptors mediate hypothermia in mice (Boulay et al., 1999; Perachon et al., 2000), a predominant role of D_3 sites has been implicated in rats (Millan et al., 2000a; Chaperon et al., 2003). Further, 7-OH-DPAT elicits hypothermia upon microinjection into the IOC, a postsynaptic action unaffected by elimination of dopaminergic neurones (Barik and Beaurepaire, 1998). Nonetheless, a role of both D_3 *and* (at higher doses) D_2 sites in the antagonism of 7-OH-DPAT-induced hypothermia by S33138 is possible. Collins et al. (2005) asserted that stimulation of D_3 and D_2 receptors respectively mediates and *opposes* induction of yawns by PD128,907 in rats. However, a high dose (56.0 mg/kg, s.c.) of the D_3 antagonist, SB277,011, was needed for full blockade, while the D_2 antagonist, L741,626, which enhanced yawns, may have been "underdosed" at 1.0 mg/kg (Bristow et al., 1998; Millan et al., 2000a, 2004b, 2007; Pak et al., 2006). Moreover, dopaminergic agonists elicit yawns by postsynaptic actions in the paraventricular nucleus, a structure lacking D_3 receptors (Argiolas and Melis 1998; Chen et al., 1999; Joyce 2001); and yawns evoked by 7-OH-DPAT are unaffected by selective D_3 antagonists yet abolished by L741,626 (Millan et al., 2000a; 2004b). Accordingly, blockade by S33138 likely reflects its antagonism of D_2 receptors. DS properties of low doses of dopaminergic agonists principally reflect recruitment of *presynaptic* D_3 and/or D_2 receptors (Cory-Slechta et al., 1996; Bristow et al., 1998; Millan et al., 2000b). Inasmuch as the PD128,907 cue is resistant to selective D_3 receptor antagonists, *potent* blockade by S33138 was unexpected. Nonetheless, potent antagonism of S33138 at presynaptic D_3/D_2 sites is underpinned by electrophysiological studies (see below), and S33138 is a potent antagonist at D_3/D_2 heterodimers which may form in dopaminergic neurones (Millan et al., submitted).

Antagonist properties of S33138 at D_3/D_2 autoreceptors. Attenuation of the inhibitory influence of PD128,907 upon VTA firing by acute S33138 at doses as low as 0.004 mg/kg, i.v. mimics findings with selective D_3 antagonists: this action likely reflects blockade of D_3 autoreceptors, activation of which phasically inhibits DA release and neuronal firing (Millan et al., 2000a, c, d; 2004a; Reavill et al., 2000; Drescher et al., 2005; Roberts et al., 2006). Conversely, *full* blockade of PD128,907 by higher doses of S33138 presumably reflects additional occupation of D_2 receptors (Usiello et al., 2000). A comparable degree of "resolution" cannot be achieved in dialysis studies, but blockade by S33138 of the PD128,907-induced reduction of DA release in FCX, nucleus accumbens and striatum (Millan et al., 2000c, 2004a; Reavill et al., 2000; Roberts et al., 2006) corroborates its antagonist properties at D_3 (and D_2) autoreceptors.

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Mimicking selective D_3 antagonists, and in contrast to D_3/D_2 and preferential D_2 antagonists, S33138 neither activated the VTA nor elevated extracellular levels of DA (Millan et al., 2000c, d; Roberts et al., 2006). There are several possible explanations. *First*, D_3 autoreceptors are not tonically active: further, even when occluded by low doses of S33138, DA has access to inhibitory D_2 autoreceptors (Millan et al., 2000c, d; Sokoloff et al., 2006). *Second*, extinction of "spontaneous" coupling at constitutively-active D_2 autoreceptors may account for activation of dopaminergic projections by haloperidol, an inverse agonist (Nilsson et al., 1998; Millan et al., 2000d): the possibility that S33138 behaves as a *neutral* antagonist is under exploration. *Third*, at high doses, antagonism by S33138 of excitatory, VTA-localized 5-HT_{2A} receptors may dampen disinhibition of dopaminergic cell bodies *via* D_2 receptor blockade (Millan et al., 2000d; Minabe et al., 2001). Regardless of the underlying reasons, this observation is intriguing since increased mesolimbic DA release provoked by haloperidol may interfere with concurrent antagonism of postsynaptic D_2/D_3 receptors and underlie patient resistance.

Lack of antagonist properties of S33138 at D_1 receptors. In rats sustaining a unilateral lesion of the SNPC, D_1 receptor agonists elicit contralateral rotation, principally *via* supersensitive striatal D_1 receptors, though D_1 sites in the substantia nigra pars reticulata may also be involved (Asin and Montana, 1988; Gulwadi et al., 2001; Gerfen et al., 2002). This response is abolished by D_1 antagonists like SCH23390. Though S33138 possesses mild affinity for D_1 receptors (Millan et al., submitted), it is >100-fold less potent than SCH23390 and did not influence SKF81297-induced rotation. Accordingly, D_1 antagonism is unlikely to be an important component of its pharmacological properties.

Influence of S33138 upon noradrenergic neurones: blockade of α_{2C}-ARs. Though S33138 excited noradrenergic perikarya (Millan et al., 2000d; Invernizzi and Garattini, 2004), D_3/D_2 receptors are unlikely to be involved since active doses were substantially higher than those blocking the influence of PD128,907 on dopaminergic neurones. In addition, selective D_3 receptor antagonists do not excite the LC (Millan et al., 2000c). Rather, antagonist properties of S33138 at α_{2C}-AR autoreceptors may be implicated inasmuch they inhibit adrenergic cell firing (Arima, 1998; Millan et al., 2000d; Owesson et al., 2003). Nonetheless, the response of the LC to *agonists* is mainly tranduced *via* α_{2A}-ARs (Mateo and Meana, 1999; Pudovkina et al., 2001). Accordingly, the lack of affinity of S33138 for α_{2A}-ARs accounts for its *inability*, in distinction to idazoxan, to prevent the suppressive influence of clonidine upon LC firing. These electrophysiological data provide a "surrogate" measure of the ability of S33138 to block *postsynaptic* populations of α_{2C}-ARs inhibitory to motor function, mood and cognition (Svensson, 2003).

Antagonist properties of S33138 at 5-HT_{2A} and 5-HT_7 receptors. Since D_3 receptor antagonists do not affect DOI-induced HTW, the modest antagonist properties of S33138 at 5-HT_{2A} receptors (Millan et al., submitted) account for its inhibition of DOI-evoked HTW which are abolished by

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selective 5-HT_{2A} receptor antagonists, like MDL100,907 (Schreiber et al., 1995). 5-HT_{2A} sites mediating HTW, are localized in the FCX (Willins and Meltzer, 1997), a structure implicated in the mood and cognitive deficits of schizophrenia, and 5-HT_{2A} receptor blockade contributes to the atypical profile of certain antipsychotics, including their low EPS potential (Meltzer et al., 2003). Activation of hypothalamic 5-HT_7 sites elicits hypothermia, an effect blocked by the selective antagonist, SB269,970 (Hagan et al., 2000; Guscott et al., 2003). Correspondingly, prevention of 5-CT-induced hypothermia by S33138 supports its abrogation of h5-HT_7 receptor-coupled cAMP formation (Millan et al., submitted). While blockade of 5-HT_7 receptors is unlikely to control psychosis *per se* (Pouzet et al., 2002; Meltzer et al., 2003), it may exert a beneficial effect on co-morbid symptoms such as depressed mood and poor sleep (Hedlund and Sutcliffe, 2004).

General discussion and conclusions. In line with cellular studies (Millan et al., submitted), these data demonstrate modest antagonist properties of S33138 at central α_{2C}-ARs, 5-HT_{2A} receptors and 5-HT_7 receptors. Most importantly, in line with antagonist properties at human D_3 and D_{2L}/D_{2S} receptors, they demonstrate that S33138 blocks cerebral populations of pre and postsynaptic D_3 and D_2 receptors. Further, mimicking *in vitro* studies, the present *in vivo* observations support preferential actions of S33138 at D_3 *vs* D_2 receptors in the CNS. Thus, doses of S33138 inducing c-fos in the D_3 receptor rich IOC were 16-fold lower than those elevating c-fos in striatum, and a similar ratio was seen for doses of S33138 which reduced motor actions of ropinirole in Parkinsonian primates *vs* those which facilitated its actions. Though comparisons are less easy to make, the more potent inhibition by S33138 of the spontaneous firing of neurons in the VTA *vs* SNPC is also consistent with more marked D_3 receptor antagonism. A limitation of these analyses is, of course, the contrasting routes, species and treatment-durations employed, all inherent to the various procedures. Furthermore, while PET studies of S33138 in man show dose-dependent (10-70 mg, p.o.) occupation of [^{11}C]raclopride-labelled D_2/D_3 sites in basal ganglia (unpub. obs.), radiolabelled antagonists differentiating D_3 and D_2 receptors are unavailable. Studies in mice genetically lacking D_3 or D_2 receptors would be useful in further characterising the roles of D_3 *vs* D_2 receptors in the actions of S33138. However: certain procedures *cannot* be performed in this species; "knock-out" mice have their own limitations; and information on novel drugs from *non*-genetically modified animals is most relevant to their potential actions in man. Finally, it would be interesting to evaluate the actions of other antipsychotics at cerebral D_3 vs D_2 receptors in the procedures employed herein.

Thus, further study of the roles of D_3 and D_2 receptors in the actions of S31338 would be instructive, but the present *in vivo* and *in vitro* studies collectively suggest that it acts as a preferential - *not* selective - antagonist of D_3 *vs* D_2 receptors. These observations provide an instructive framework for interpreting its potential antipsychotic properties in experimental studies and in ongoing (Phase II) clinical trials in man.

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LEGENDS FOR FIGURES

Figure 1. Influence of S33138 upon c-fos gene expression in mice.

The influence of S33138 (i.p.) is expressed relative to basal values (100 %). They were (in arbitrary units of optical density), 5.6 ± 1.3, 1.2 ± 2.6 and 22.1 ± 5.2 for the Isles of Calleja, nucleus accumbens and striatum, respectively. Data are means ± SEMs. N = 6-8 per value. ANOVA as follows: Isles of Calleja, $F(4,35) = 8.5$, $P < 0.01$; nucleus accumbens, $F(4,35) = 7.4$, $P < 0.01$ and striatum, $F(4,35) = 18.9$, $P < 0.01$. Asterisks indicate the significance of differences of S33138 *vs* vehicle values in Dunnett's test. * $P < 0.05$.

Figure 2. Representative microphotographs showing the induction by S33138 of cerebral c-fos gene expression in mice.

Emulsion-dipped sections from an *in situ* hybridization experiment show the expression of c-fos mRNA in the Isles of Calleja (upper panels), nucleus accumbens (middle panels) and striatum (lower panels) in response to vehicle, S33138, 0.16 mg/kg, i.p. and S33138, 2.5 mg/kg, i.p.. Silver grains correspond to c-fos expression.

Figure 3. Influence of long-term (3 weeks) p.o. administration of S33138 upon spontaneously-active dopaminergic neurons in anesthetized rats.

Panel A, Ventral tegmental area (VTA) and Panel B, Substantia nigra, pars compacta (SNPC). Data are means ± SEMs of the number of spontaneously-active neurons detected upon stereotaxic descent. N = 10 per value. ANOVA as follows: VTA, $F(4,44) = 21.2$, $P < 0.01$ and SNPC, $F(4,44) = 8.1$, $P < 0.01$. Asterisks indicate significant difference ($P < 0.05$) from vehicle values in Newman-Keuls test following ANOVA.

Figure 4. Modulation by S33138 of the influence of ropinirole upon motor activity of parkinsonian primates pre-treated with MPTP.

Panel A, Dose-dependent influence of S33138 (p.o.) upon the action of ropinirole (0.63 mg/kg, p.o.) over 120 min and Panel B, Time-course of the influence of S33138 (0.16 mg/kg, p.o). In Panel A, data are means ± SEMs and, in Panel B, means only are shown for clarity. N = 6 per value. ANOVA (Panel A) as follows: $F(6,30) = 6.6$, $P < 0.01$. Asterisks indicate significant differences of S33138/ropinirole *vs* vehicle/ropinirole values in paired t-tests ($P < 0.05$). For Panel B, 2-way ANOVA with a repeated measure on time: ropinirole x S33138 x time, $F(24,480) = 6.4$, $P < 0.01$. Asterisks indicate significance of S33138/ropinirole *vs* vehicle/ropinirole

and vehicle/ropinirole *vs* vehicle/vehicle differences in Bonferroni's tests, $P < 0.05$.

Figure 5. Antagonism by S33138 of the actions of 7-OH-DPAT and PD128,907 in rats.

Inhibition by S33138 (s.c.) of: Panel A, Induction of hypothermia by 7-OH-DPAT (0.16 mg/kg, s.c.); Panel B, induction of yawning by 7-OH-DPAT (0.04 mg/kg, s.c.) and Panel C, discriminative stimulus properties of PD128,907 (0.16 mg/kg, i.p.). In panels A and B, data are means ± SEMs. N = 5-8 per value. Panel A, open asterisks indicate the significance of vehicle/7-OH-DPAT *vs* vehicle/vehicle values ($P < 0.01$, two-tailed t-test). ANOVA as follows: S33138/vehicle, $F(4,20) = 0.2$, $P > 0.05$ and S33138/7-OH-DPAT, $F(4,21) = 4.2$, $P < 0.01$. Panel B: ANOVA as follows: S33138, $F(4,41) = 6.3$, $P < 0.01$. In Panels A and B, closed asterisks indicate the significance of S33138/7-OH-DPAT *vs* vehicle/7-OH-DPAT values in Dunnett's test. * $P < 0.05$. In panel C, data are percentage of animals selecting the "PD128,907" lever. N = 5-6 per value. Asterisks indicate significance of S33138 *vs* control values (100 %) in Fisher Exact Probability tests (* $P < 0.05$).

Figure 6. Antagonism by S33138 of the inhibitory influence of PD128,907 upon the firing rate of ventral tegmental area dopaminergic neurones in anesthetised rats.

Panel A, Dose-dependent reversal by S33138 (i.v.) of the PD128,907 (0.01 mg/kg, i.v.) induced inhibition of ventral tegmental dopaminergic neurones. Panel B, Influence of S33138 upon firing rate, and prevention of the inhibitory influence of apomorphine (APO). Panels C and D are representative recordings of the influence of S33138 upon the actions of PD128,907 and apomorphine, respectively. For panels A and B, drug actions are expressed relative to baseline values (0 %). Data are means ± SEMs, N = 5-6 per value. Panel A. PD128,907 *vs* vehicle, $F(1,64) = 483.6$, $P < 0.01$; S33138, $F(5,33) = 1.92$, $P > 0.05$ and interaction $F(5,64) = 20.1$, $P < 0.01$. The asterisks indicate the significance of PD128,907/vehicle *vs* vehicle/vehicle and of PD128,907/S33138 *vs* PD128,907/vehicle values in Newman-Keuls test ($P < 0.05$). Panel B, $F(6,54) = 2.78$, $P < 0.05$. $P < 0.05$ *vs* vehicle in Dunnett's test.

Figure 7. Blockade by S33138 of the inhibitory influence of the preferential D_3 *vs* D_2 agonist, PD128,907, upon extracellular levels of dopamine in freely-moving rats.

Panel A, Influence of S33138 (s.c.) upon the action of PD128,907 in frontal cortex; Panel B, Influence of S33138 alone in frontal cortex: Panel C, Action of S33138 in the nucleus accumbens and Panel D, Action of S33138 in the striatum. Data (means ± SEMs) are expressed relative to basal, pre-injection values (defined as 100 %). N = 5-12 per value. Absolute, basal levels of DA were 0.97 ± 0.05 pg/20 µl, 0.94 ± 0.15 and 11.98 ± 0.76 pg/20 µl for frontal

cortex, nucleus accumbens and striatum, respectively. Panel A, ANOVA follows: 0.16 mg/kg, $F(1,15) = 0.7$, $P > 0.05$; 0.63, $F(1,16) = 22.0$, $P < 0.01$ and 2.5, $F(1,16) = 27.6$, $P < 0.01$; Panel B, ANOVA as follows: 0.16 mg/kg, $F(1,10) = 0.1$, $P > 0.05$; 0.63, $F(1,11) = 0.3$, $P > 0.05$ and 2.5, $F(1,11) = 2.8$, $P > 0.05$; Panel C, ANOVA as follows: PD128,907, $F(1,14) = 8.4$, $P < 0.05$; S33138, $F(1,12) = 0.3$, $P > 0.05$ and interaction, $F(1,13) = 5.5$, $P < 0.05$ and Panel D, ANOVA as follows: PD128,907, $F(1,14) = 19.0$, $P < 0.05$; S33138, $F(1,12) = 5.8$, $P < 0.05$ and interaction, $F(1,13) = 557$, $P < 0.05$. Open asterisks indicate significance ($P < 0.05$) of vehicle/PD128,907 *vs* vehicle/vehicle values, and closed asterisks, S33138/PD128,907 *vs* vehicle/PD128,907 values. * $P < 0.05$.

Figure 8. Antagonist actions of S33138 at α_{2C}-adrenoceptors (ARs), 5-HT_{2A} receptors and 5-HT_7 receptors.

Panel A, Influence of S33138 (i.v.) upon the firing rate of adrenergic and serotonergic neurones in the locus coeruleus (LC) and dorsal raphe nucleus (DRN), respectively. N = 5-6 per value. ANOVA as follows: LC: $F(6,46) = 5.4$, $P < 0.01$ and DRN, $F(6,44) = 0.6$, $P > 0.05$. For the LC, administration of S33138 was followed by a single dose (0.01 mg/kg, i.v.) of the α_2-AR agonist, clonidine, which significantly ($P < 0.05$, paired t-test) reduced the firing rate of adrenergic neurones. In the DRN, administration of S33138 was followed by a single dose (0.005 mg/kg, i.v.) of the 5-HT_{1A} receptor agonist, 8-OH-DPAT, which significantly ($P < 0.05$, paired t-test) attenuated the firing rate of serotonergic neurones. Panel B, Influence of S33138 (s.c.) upon the induction of head-twitches in rats by the 5-HT_{2A} agonist, DOI (2.5 mg/kg, i.p.). N = 6-7 per value. $F(4,33) = 6.7$, $P < 0.01$. Panel C, Influence of S33138 (i.p.) upon induction of hypothermia in guinea pigs by the "5-HT_7"agonist, 5-carboxytryptamine (5-CT, 3.0 mg/kg, i.p.). N = 4-10 per value. The open asterisk indicates the significance of vehicle/5-CT *vs* vehicle/vehicle values ($P < 0.01$, two-tailed t-test). ANOVA as follows: S33138/vehicle, $F(3,20) = 1.7$, $P > 0.05$ and S33138/5-CT, $F(3,20) = 7.5$, $P < 0.01$. All data are means ± SEMs. Closed asterisks indicate significant differences of S33138 *vs* vehicle values in Dunnett's test. * $P < 0.05$.

Figure 1

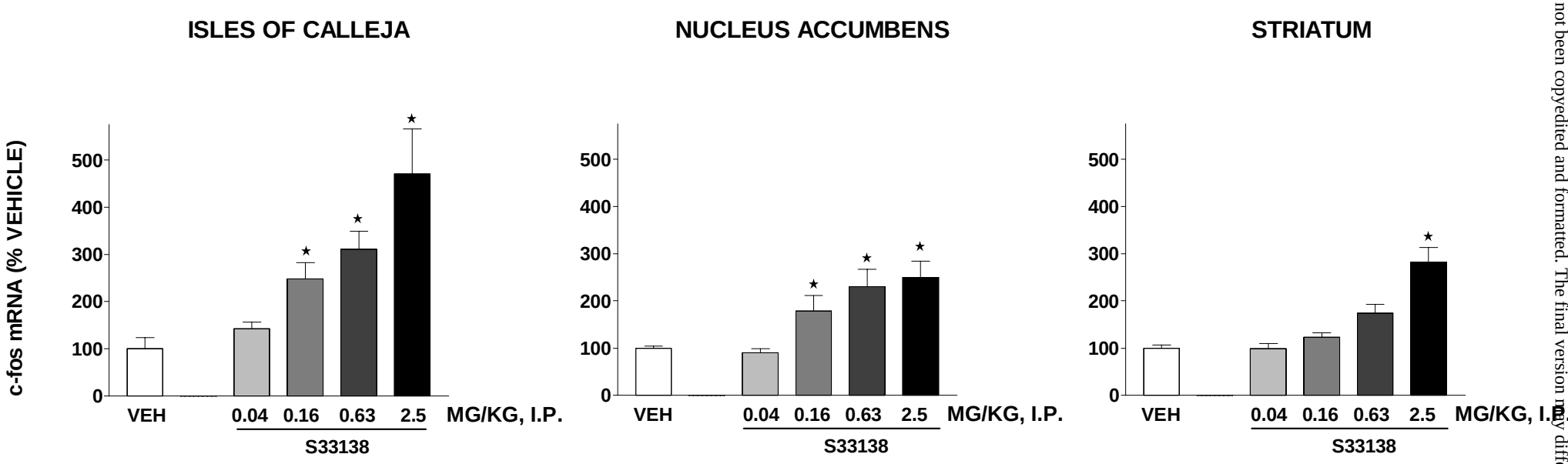


Figure 2

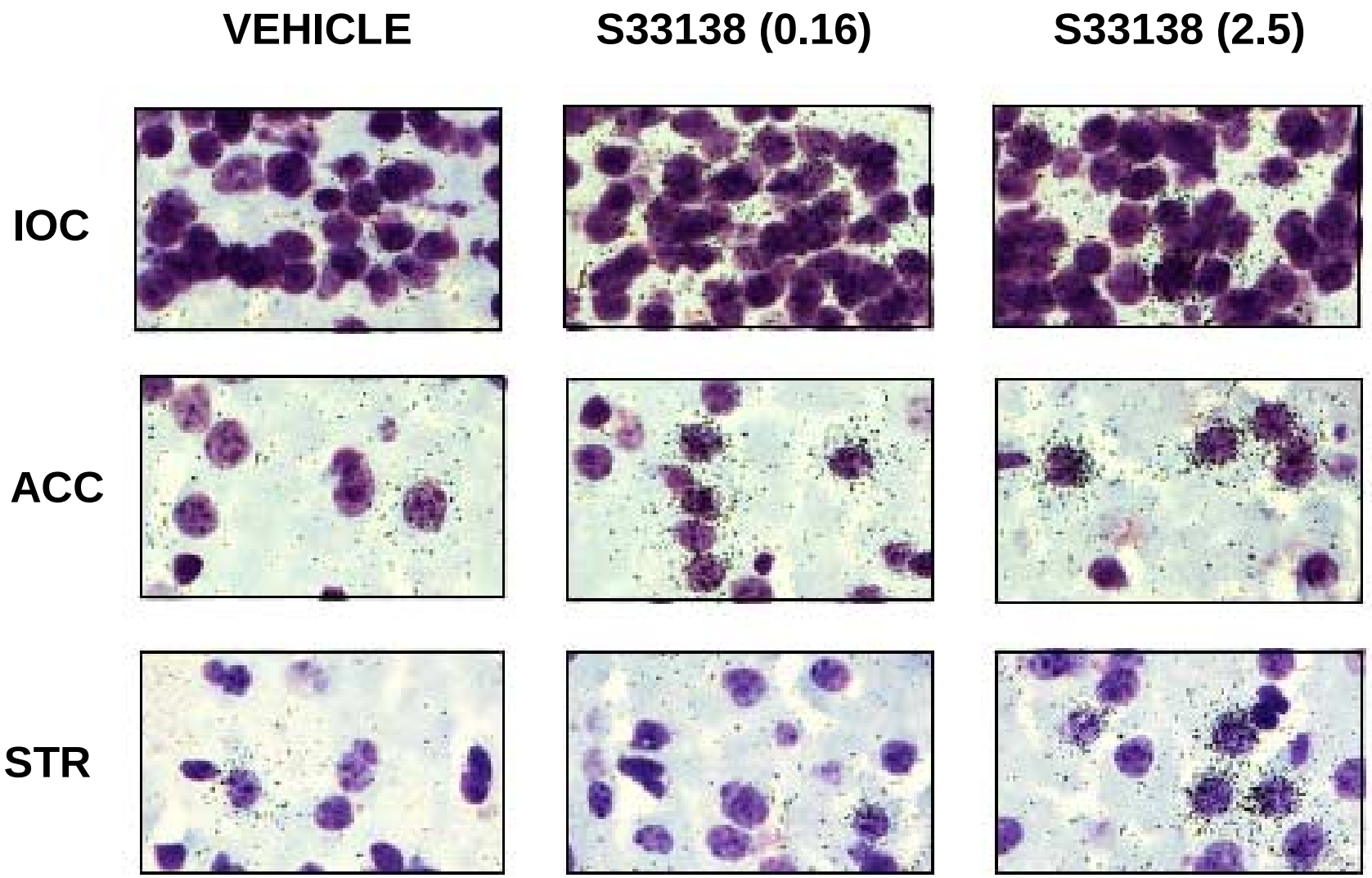


Figure 3

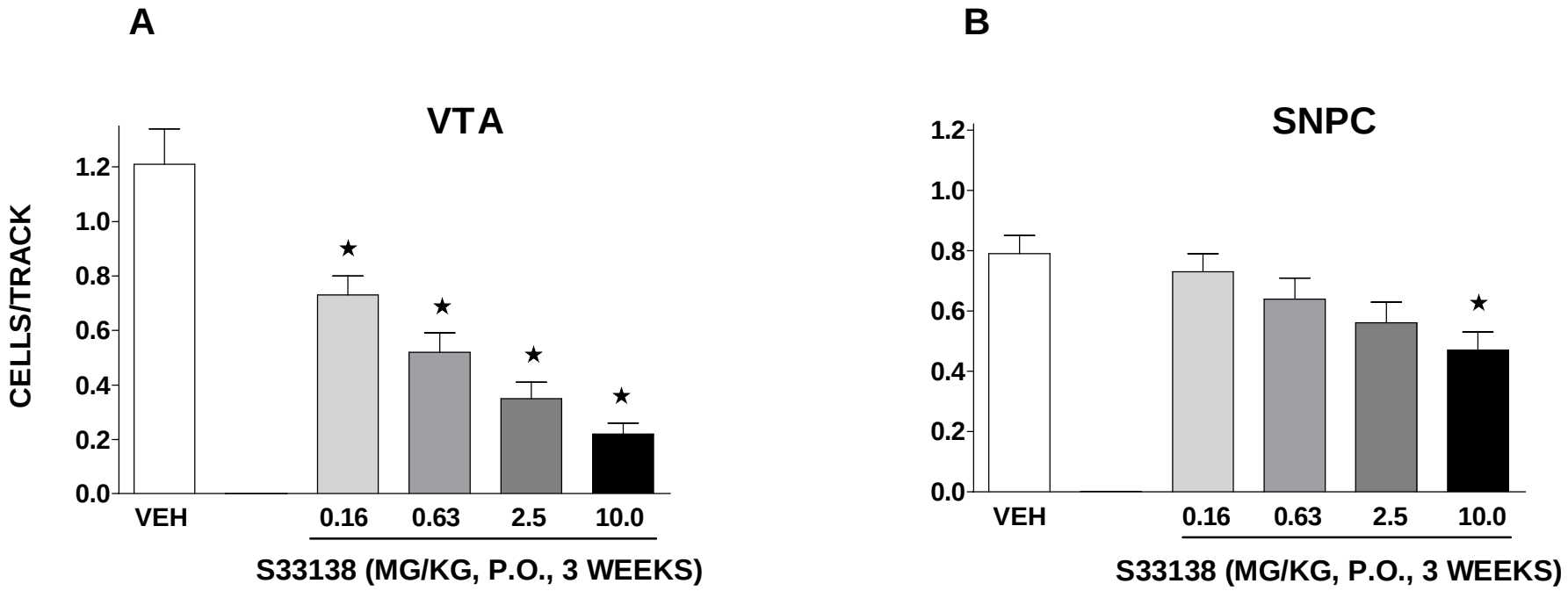
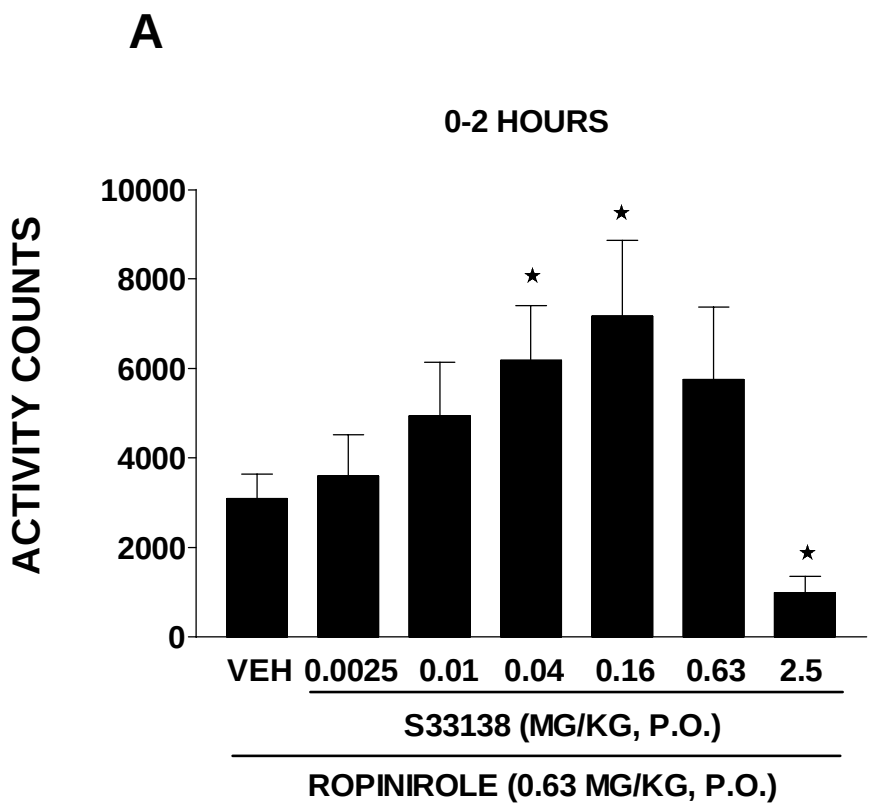


Figure 4



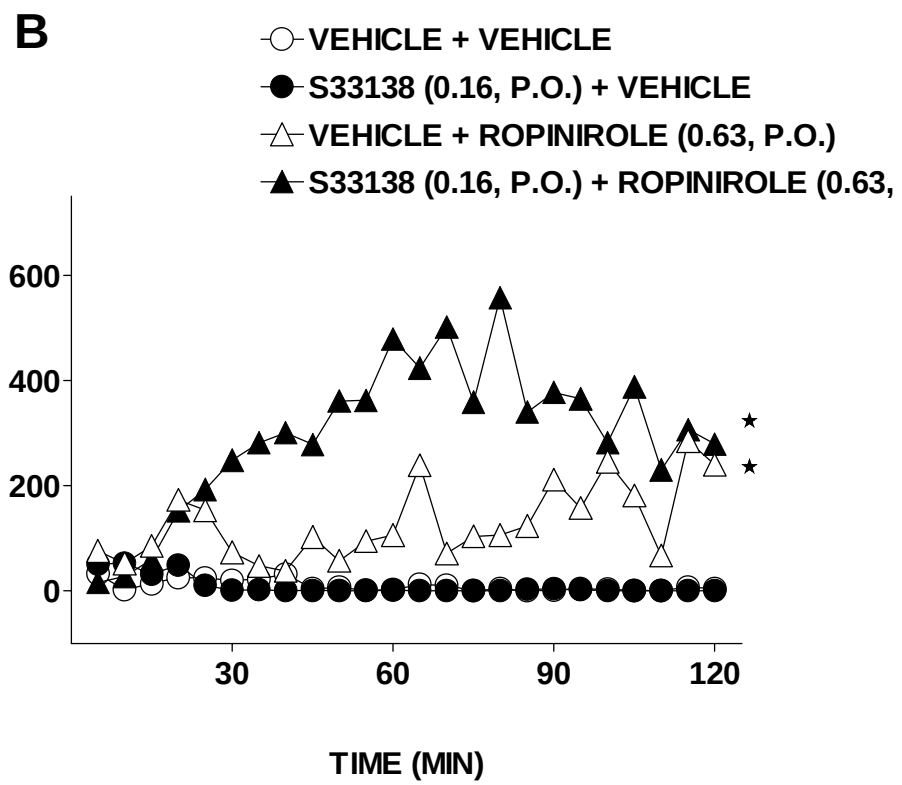


Figure 5

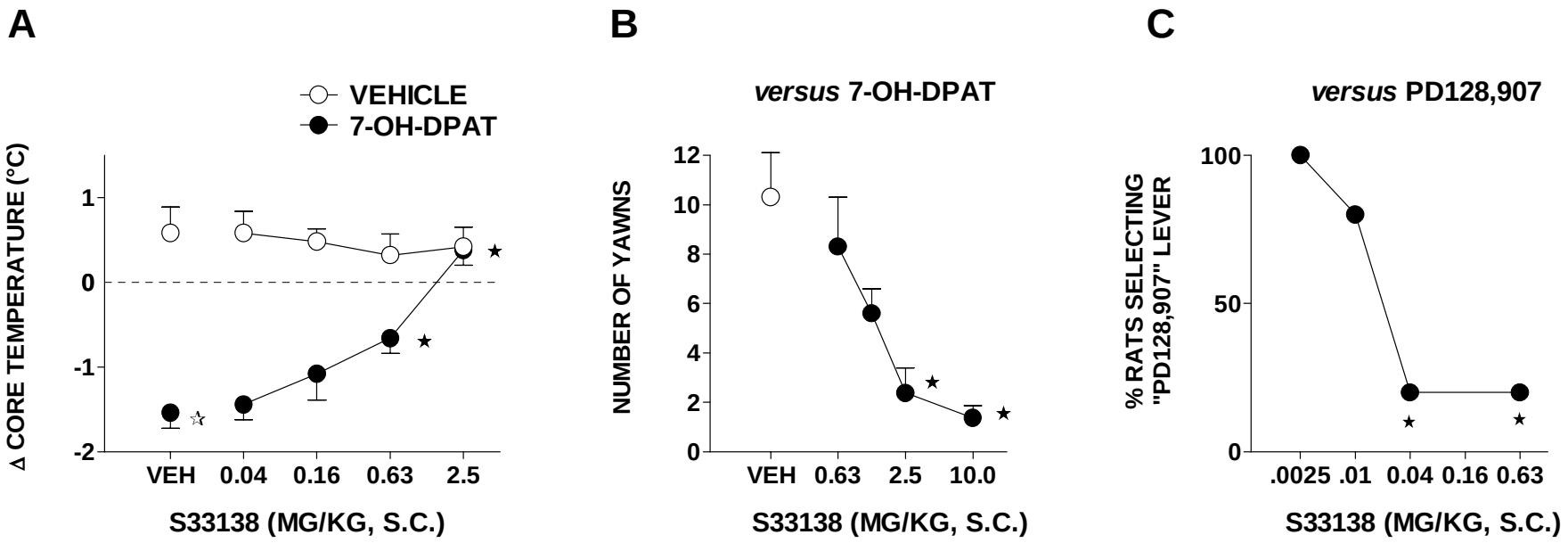


Figure 6

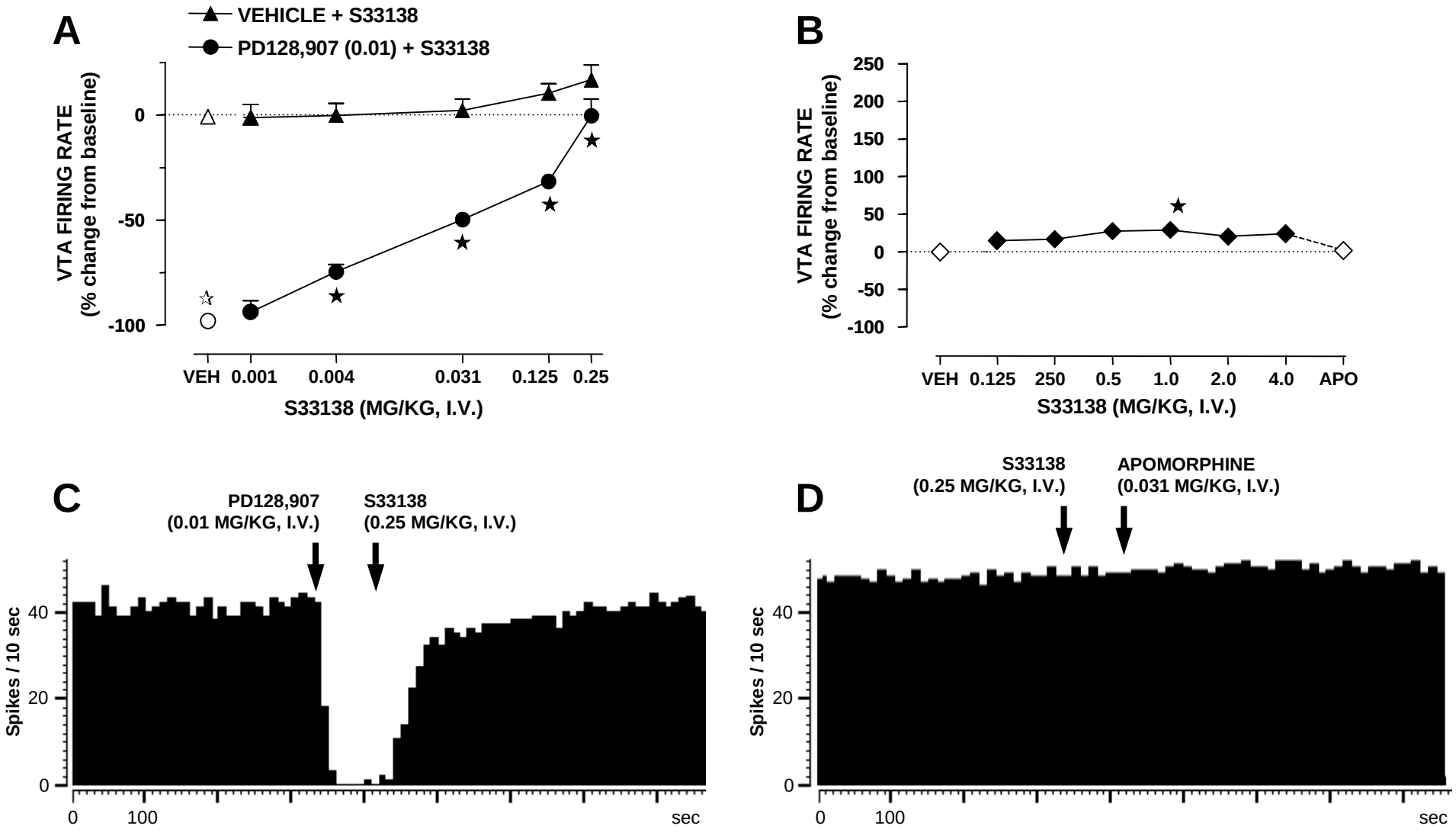


Figure 7

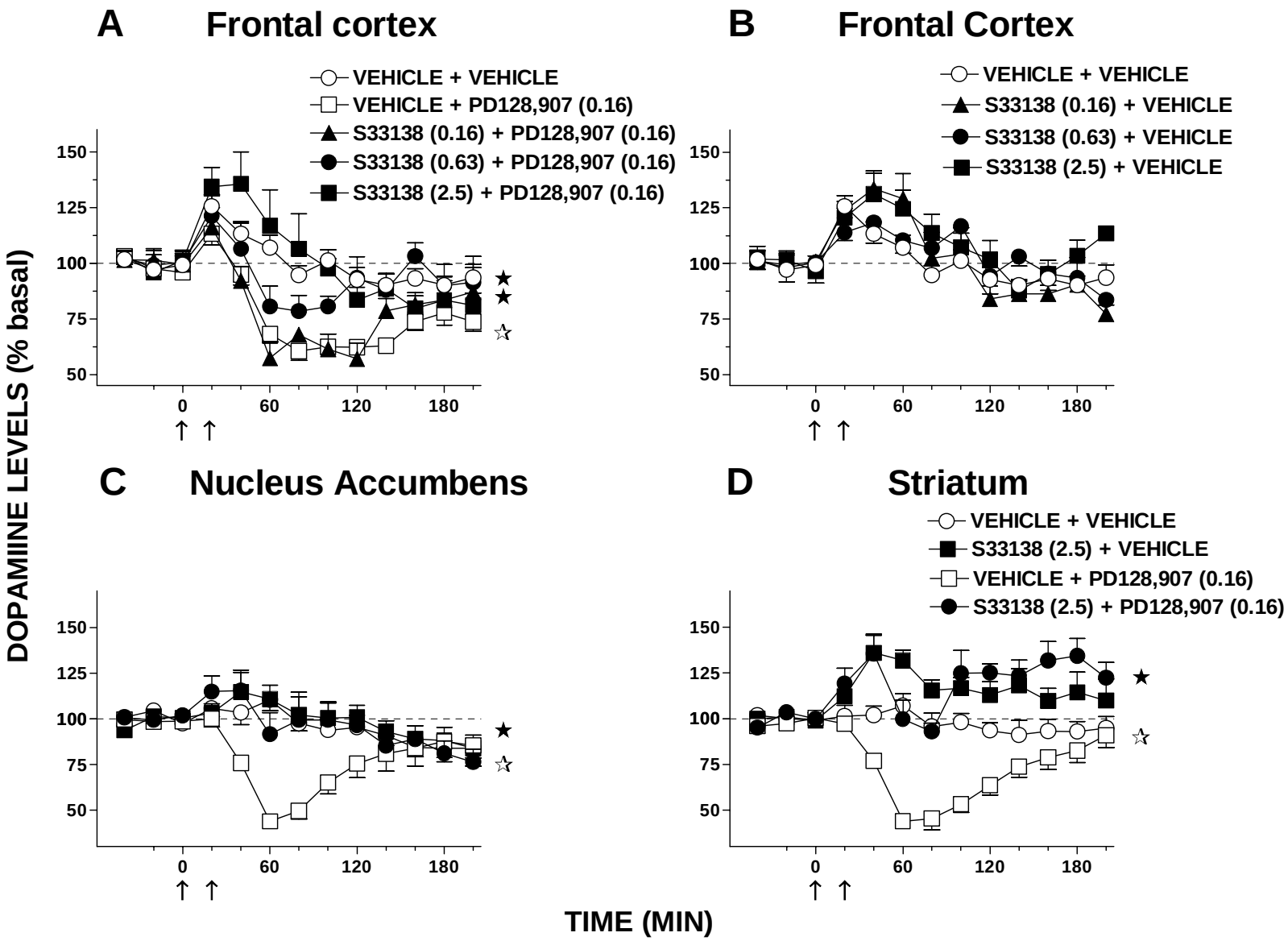


Figure 8