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Differences between A 68930 and SKF 82958 could suggest synergistic roles of D_1 and D_5 receptors[☆]

R. Nergårdh [a,b,*], S. Oerther [a,c], B.B. Fredholm [a]

[a] *Department of Physiology and Pharmacology, Section of Molecular Pharmacology, Karolinska Institutet, S-171 77 Stockholm, Sweden*
[b] *Department of Neurotec, Section of Applied Neuroendocrinology, Karolinska Institutet, Huddinge University Hospital, S-141 86 Huddinge, Sweden*
[c] *AstraZeneca Research and Development, Södertälje, Sweden*

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Abstract

The isochroman A 68930 and the benzazepine SKF 82958 are two full dopamine D_1 receptor[1] agonists. Responses to these compounds are different in several important aspects.

When given to rats in a novel environment, A 68930 caused a dose-dependent (0.019–4.9 mg/kg) suppression of locomotion. SKF 82958 had no such effect at any dose studied (0.051–3.3 mg/kg). In animals habituated to the environment, A 68930 had no effect but SKF 82958 increased locomotor activity. Both A 68930 and SKF 82958 caused a decrease in core temperature at early time points. Both agonists increased c-*fos* and NGFI-A expression in caudate putamen but only SKF 82958 did so in the accumbens nucleus (at 1.6 mg/kg). Quantitative receptor autoradiography showed that A 68930 is 9–13 times more potent than SKF 82958 at displacing the selective dopamine D_1 antagonist [^{3}H]SCH 23390. This difference agrees with the difference observed when the agonists were used to stimulate cAMP formation in cells transfected with the D_1 receptor. In contrast, SKF 82958 was 5 times more potent than A 68930 in cells transfected with the D_5 receptor.

We suggest that the balance between signaling via dopamine D_1 and D_5 receptors determines the functional effects of agonists at D_1/D_5 receptors.
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Keywords: Dopamine; Dopamine D_1 receptors; Dopamine agonists; Immediate early gene; Locomotion; In situ hybridization

1. Introduction

Dopamine (DA) exerts its effects via five different G protein coupled receptors (D_1 through D_5) (Schwartz et al., 2000). They can be grouped into D_1-like or D_2-like depending on their ability to increase or decrease cAMP formation, respectively (Gingrich and Caron, 1993). The D_1 receptor subfamily has two members, the D_1 and D_5 receptors. There are several agonists and antagonists that can discriminate between the D_1-like and D_2-like receptors, but few agents are selective for individual members of the DA D_1 family of receptors. There are also growing numbers of observations showing behavioral differences between supposed DA D_1 family selective agonists (Desai et al., 2003; Makihara et al., 2004). The isochroman (1*R*,3*S*)-1-aminomethyl-5,6-dihydroxy-3-phenylisochroman HCl (A 68930) and the benzazepine (±)-6-chloro-7,8-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine HBr (SKF 82958) are two highly selective DA D_1 receptor agonists with full efficacy as regard to cAMP formation (DeNinno et al., 1991; Svenningsson et al., 2000). These two compounds differ markedly, however, in their reported ability to affect spontaneous locomotor activity. A 68930 decreases spontaneous locomotor activity (Salmi and Ahlenius, 2000) whereas SKF 82958 stimulates it (Le Moine et al., 1997). SKF 82958 has

[☆] This paper is dedicated to the memory of Professor Sven Ahlenius who died the 19th of February 2001 before the completion of this study.

* Corresponding author. Department of Physiology and Pharmacology, Section of Molecular Pharmacology, Karolinska Institutet, S-171 77 Stockholm, Sweden. Tel.: +46 8 5248 7941; fax: +46 8 34 12 80.
E-mail address: Ricard.Nergardh@fyfa.ki.se (R. Nergårdh).

[1] In this paper we have used the D_1 through D_5 nomenclature in accordance with IUPHAR recommendations (Schwartz J-C, Carlsson A, Caron M, Scatton B, Civelli O, Kebabian JW et al (2000). Dopamine Receptors. The IUPHAR Compendium of Receptor Characterization and Classification. Anonymous. IUPHAR Media: London. pp 171-181.) despite the fact that for rodent receptors D_1 receptors are often referred to as D_{1a} and D_5 receptors as D_{1b}.

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also shown several behavioral and pharmacological differences compared to other D_1 family selective agonists. (Desai et al., 2003; Makihara et al., 2004) In the present paper, we have examined the effects of these two agonists on spontaneous locomotor activity of rats in an open-field arena. Since it is well known that DA D_1 receptor stimulation has effects on core temperature (Clark and Lipton, 1985), we used this as an autonomic readout of DA receptor activation and compared the two agonists in this regard as well. We have related these actions to expression of immediate early genes (IEG) in the rat brain to look for differences in regions activated after treatment with any of the two compounds. To characterize the pharmacological differences, we performed quantitative receptor autoradiography on brain slices.

Different distribution (Khan et al., 2000; Luedtke et al., 1999; Sibley, 1999) and differences in the phenotype of D_1 and D_5 knock-out mice (Holmes et al., 2001; Waddington et al., 2001) indicate functionally different roles for the members of the DA D_1 receptor family (Waddington et al., 1995, 2001; Nicola et al., 2000).

A 68930 remains effective, albeit with some effects altered, in D_1 null mutant mice (Clifford et al., 1999) and SKF 82958 shows psychopharmacological distinctions compared to other D1 family agonists (Deveney and Waddington, 1997). In view of the fact that A 68930 and SKF 8295 act not only on D_1 but also on D_5 receptors we used cAMP formation in Chinese hamster ovary cells (CHO cells) transfected with either the D_1 or the D_5 receptor as a functional assay to confirm that the compounds are full agonists and also to assess their relative potency on the two types of receptors.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats (280–320 g) were used (B and K Universal AB, Sollentuna, Sweden). The animals arrived in the laboratory at least 10 days before being used in experiments and were housed, five per cage (Makrolon IV), under controlled conditions of temperature (21.0±0.4 °C), relative humidity (55–65%) and light–dark cycle (12:12 h, lights off 06.00 Hillegaart and Ahlenius, 1994). Food (R36, Ewos, Södertälje, Sweden) and tap water were available ad libitum in the home cage. The experiments were conducted according to EU rules, and were approved by the regional animal ethics committee.

2.2. Drugs

(1*R*,3*S*)-1-aminomethyl-5,6-dihydroxy-3-phenylisochroman HCl (A 68930; A-75734.1), mol. wt. 307.6 (Abbott, Abbott Park, IL) and (±)-6-chloro-7,8-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine HBr (SKF 82958), mol. wt. 410.7 (RBI, Natick, MA) were dissolved in physiological saline and injected subcutaneously in a volume of 2 mL/kg. Controls received the saline vehicle.

2.3. Observation of spontaneous motor activity and core temperature

Spontaneous motor activity was observed in a square open-field arena (680×680×450 mm), equipped with two rows of photocells (8×8), sensitive to infrared light (Regler och mätteknik, Kungsbacka, Sweden). Two identical frames of photocells were placed at two levels, 40 and 125 mm above the floor. The photocells were spaced 90 mm apart, and the last photocell in a row was 25 mm from the wall. The open-field arena was enclosed in a ventilated, sound-insulated box with a Perspex top. Measurements were made in the dark between 09.00 and 16.00 h and data were collected during a 15-min long test session 20 min after drug administration.

The number of photocell beam interruptions was collected on a PC, and the following variables were calculated: locomotor activity (all interruptions of photobeams at the lower level); peripheral locomotion (interruptions of photobeams provided that the photobeams spaced 25 mm from the wall at the lower level also had been activated); rearing (all interruptions of photobeams at the upper level); forward locomotion (successive interruptions of photocells in the lower rows when the animal was moving in the same direction).

Locomotor activity and rearing data were subjected to a square root transformation. Peripheral locomotion and forward locomotion are expressed as percent of total horizontal activity and the quotients were based on raw data. For further details on the apparatus and the computer software used, see Ericson et al. (1991). Separate groups of animals for the two conditions ("naïve" and "habituated"), and doses of A 68930 or SKF 82958, were used for the open-field observations. In the experiments using habituated rats, the rats were habituated to the open-field arena for 20 min. The "naïve" animals were also used for the temperature experiments.

Core temperature measurements were made in a temperature-controlled room (ambient temperature 21.0±0.4 °C). Recordings were made by means of a commercially available telethermometer (YSI-2100, Yellow Springs Instruments Co, Yellow Springs, OH) and an accompanying probe (YSI-402). The probe, lubricated with mucilago etalosi AF-68 (ACO Läkemedel AB, Stockholm, Sweden), was inserted rectally (about 90 mm) in the gently hand-restrained rat. The telethermometer was connected to an automatic printer device that was activated when the temperature reading had stabilized (±0.4 °C) for 10 s. For further details on the apparatus used, see Salmi et al. (1994). A first reading of core temperature was taken immediately before the animals were placed in the open-field arena, 20 min after injection of A 68930 or SKF 82958, and a second reading of the same animals was made at 60 min after drug injection. For comparison, core temperature was measured in unhabituated, untreated rats at the same time points.

Parametric statistical procedures for description and analysis were used throughout. Thus, results are presented as means±S.E.M., and a one-way ANOVA, followed by Dunnett's test, was used to analyze the dose–effect curves for the respective dopamine D_1 receptor agonists.

2.4. Analysis of immediate early gene expression

Two hours after drug treatment the animals were briefly anesthetized with CO_2 and killed immediately by decapitation. Brains were rapidly dissected out and frozen on dry ice. Brains were then stored at −80 °C until sectioning. Coronal sections (14 μm for exposure on films and 6 μm for co-localization studies) were made through striatum at levels bregma 1.6 mm and bregma −0.92 mm according to the atlas by Paxinos and Watson (1998). Sections were thaw-mounted on poly-L-lysine coated slides and stored at −20 °C until hybridization.

For in situ hybridization the following probes were used: c-*fos*, 45 bases long, complementary to rat c-*fos* mRNA encoding amino acid 137–152 of the c-Fos protein (Curran et al., 1987); NGFI-A, 45 bases long, complementary to rat NGFI-A mRNA encoding amino acids 2–16 of the NGFI-A protein (Milbrandt, 1987), preproenkephalin (Enk), 48 bases long, complementary to nucleotides 388–435 of the rat preproenkephalin gene (Yoshikawa et al., 1984). In situ hybridization was performed as described by Svenningsson et al. (1998) and sections were exposed on Hyperfilm β-max (Amersham Pharmacia Biotec Inc.) for 4 weeks. To examine co-localization of IEG expression and preproenkephalin mRNA, consecutive sections were hybridized with the c-*fos* probe or the Enk probe. Sections were then dipped in emulsion (Amersham Pharmacia Biotec Inc.) and exposed for 20 weeks.

The autoradiographic films were analyzed on a PC-based system running Scion Image. The system was calibrated using an external Kodak density wedge. Mean optical density in a given region was measured. Data are expressed as means ± S.E.M. for each group. Since the two D_1 agonists were given on different days the A 68930 and the SKF 82958 treated animals had their own saline-treated control groups. All the saline-treated control animals from the two experimental days were then pooled. Measurements from caudate putamen (CPu) were performed on two levels corresponding to bregma 1.6 mm and bregma −0.92 mm according to the Paxinos and Watson atlas . Measurements from accumbens nucleus were performed in the core (AcbC) and shell (AcbSh) part separately at a level corresponding to bregma 1.6 mm according to the Paxinos and Watson atlas. Parametric statistical procedures were used throughout. One-way ANOVA followed by Bonferroni's multiple comparison test was used to analyze the differences in optical density between the groups. A p-value <0.05 was considered significant. To compare the differences in amplitude of the IEG induction in AcbC and AcbSh a two-way ANOVA was performed. For all calculations GraphPad Prism (version 3.00 for Windows, GraphPad Software, San Diego, CA, USA) was used. For co-localization studies we used a Zeiss Axioscope 2 microscope with a digital Axio Cam attached to it. Consecutive 6 ìm sections hybridized for Enk or NGFI-A were compared based on morphology and individual neurons appearing on both sections were identified. Cholinergic interneurons were identified by size.

2.5. Receptor autoradiography

To construct competitive binding curves against [^{3}H]SCH 23390, autoradiographic experiments were done. Two rats were anesthetized with CO_2 and killed immediately by decapitation. Brains were rapidly dissected out and frozen on dry ice. Brains were then stored at −80 °C until sectioning. Coronal sections 14 μm were made through striatum at the same levels as used in the in situ hybridization studies. Sections were thaw-mounted on poly-L-lysine coated slides and stored at −20 °C until used. On the day of the assay, slides were dried for 60 min at room temperature and then incubated with 1 nM [^{3}H]7-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepine-7-ol (NEN™ Life Science Products, Inc. Boston, MA, USA) and increasing concentrations of either SKF 82958 or A 68930 in a buffer containing 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 2.66 mM KCl, 1.35 mM $CaCl_2$, 1 μM pargyline and 0.001% ascorbic acid. Mianserin (20 nM) was also included to block binding of SCH 23390 to serotonin 5-HT_2 receptors. Sections were incubated for 60 min at room temperature. Slides were then washed twice for 5 min each in ice-cold buffer and dried at 4 °C over a strong fan over night. Slides were apposed to Hyperfilm-^{3}H (Amersham Pharmacia) for 6 months at 4 °C together with [^{3}H] standards (Amersham Pharmacia). Non-specific binding was defined by 100 μM DA.

The autoradiograms were analyzed on a PC-based system running Scion Image. Optical density values were converted to binding density (fmol/mg tissue) using the [^{3}H] standards and the specific activity of the radioligand.

2.6. Functional assay

CHO cells were grown on 6-well plates in DMEM (Dulbecco's modified Eagle's medium)/Hamm's F-12 medium containing 10% fetal bovine serum and 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 200 μg/ml geniticin. Subconfluent cells were transfected with hD_1 and hD_5 cDNA (Jarvie et al., 1993, kindly provided by Dr. Marc Caron; Gingrich et al., 1992). Cells were left overnight in the same medium as before transfection and stimulated with dopamine or one of the two D_1 agonists the following day. Prior to assay the cells were washed twice with HEPES-buffered (20 mM) DMEM and pre-incubated in 225 μl of the same medium at 37 °C for 30 min. Drugs to be tested (i.e., A 68930 and SKF 82958) were dissolved in 25 μl of the same medium and added to each well to a final volume of 250 μl. Dopamine was dissolved in 25 μl of the same medium with 20 μg/ml ascorbic acid added. After 20 min of incubation at 37 °C the reaction was stopped by addition of perchloric acid to a final concentration of 0.4 M. The cell suspension was kept on ice for 1 h and then neutralized with potassium hydroxide in Tris and centrifuged. The cAMP concentration in the supernatant was determined using a competitive binding assay (Nordstedt and Fredholm, 1990) modified for 96-well format.

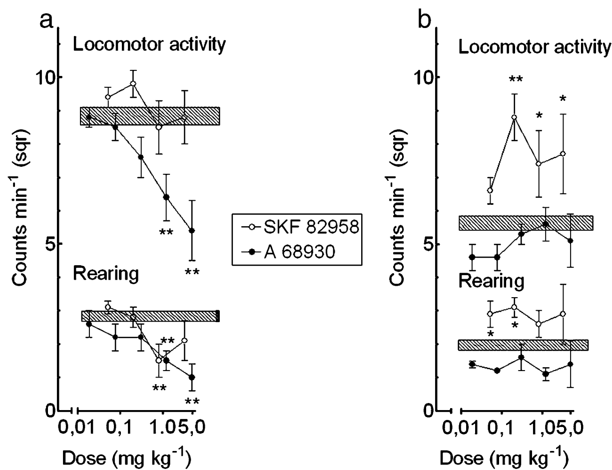

Fig. 1. Effects of A 68930 and SKF 82958 on motor activity and rearing in rats naïve (a) or habituated (b) to an open field. Results are presented as means ± S.E.M., based on observations of 4–6 rats per group. The pooled vehicle-treated controls ($n = 13$) are shown by the shaded area. A 68930 produced a dose-dependent (0.019–4.9 mg/kg) suppression of locomotor activity and rearing in naïve animals (a). SKF 82958 had no effect on locomotor activity over a wide range of doses (0.051–3.3 mg/kg) in the naïve animals. The intermediate dose of 1.6 mg/kg SKF 82958 produced a statistically significant suppression of rearing behavior. In rats habituated to the open field (b), A 68930 (0.019–4.9 mg/kg) did not affect overall locomotor activity or rearing, whereas SKF 82958 (0.051–3.3 mg/kg) produced a statistically significant increase in locomotor activity as well as rearing behavior.

For each assay, cells were stimulated with both dopamine and agonist (i.e., A 68930 or SKF 82958). Since there are variations in transfection efficacy between assays a dose–response curve was calculated for dopamine in every assay, and that curve was used to define top and bottom values for the assay. The A 68930 and SKF 82958 curves were then normalized to those values. To be able to compare EC_{50} values between assays, the EC_{50} value for the agonist was related to the EC_{50} value for dopamine in the same assay. Dose–response curves were calculated according to the following equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / 1 + 10^{(\log EC50 - X)\text{Hillslope}}$, using GraphPad Prism (version 3.00 for Windows; San Diego, CA, USA). All results are presented as means and 95% confidence intervals to facilitate comparisons between treatments.

3. Results

3.1. Effects of A 68930 and SKF 82958 on open-field motor activity

We first wanted to confirm that the two D_1/D_5 agonists A 68930 and SKF 82958, reported in different studies to have opposite effects (Le Moine et al., 1997; Salmi and Ahlenius, 2000), do indeed differ when examined under identical conditions. Since it is well known that a novel environment has pronounced effects on locomotor activity in rats (Hillegaart and Ahlenius, 1994), we decided to test the effect of the agonists both in animals given the opportunity to habituate to the open-field arena for 15 min and in animals naïve to the open-field arena. As expected, animals pre-exposed to the open-field arena for 15 min displayed a clear and statistically significant decrease in locomotor activity and rearing behavior in comparison with animals naïve to the open field ($p < 0.01$; Student's t-test), indicating habituation.

In the naïve animals A 68930 produced a dose-dependent (0.019–4.9 mg/kg) suppression of locomotor activity (Fig. 1a) and rearing. In addition, there was a marked decrease in the proportion of forward locomotion, whereas the proportion of peripheral locomotion was unchanged in comparison with saline-treated controls (data not shown). SKF 82958, on the other hand, had no effect on overall locomotor activity over a wide range of doses (0.051–3.3 mg/kg) in the naïve animals (Fig. 1a) except for a dose-dependent decrease in the component of total locomotion that could be ascribed to forward locomotion (data not shown). The intermediate dose of 1.6 mg/kg SKF 82958 produced a statistically significant suppression of rearing behavior (Fig. 1a) as well as a decrease in the proportion of peripheral locomotion (data not shown).

In rats habituated to the open field, A 68930 (0.019–4.9 mg/kg) did not affect overall locomotor activity or rearing, whereas SKF 82958 (0.051–3.3 mg/kg) produced a statistically significant increase in locomotor activity as well as rearing behavior (Fig. 1b). Within the dose interval used here, this effect of SKF 82958 was not clearly dose-dependent and was maximal already at 0.051 mg/kg (rearing) or 0.21 mg/kg (locomotor activity). The increased locomotor activity and rearing behavior were not accompanied by any statistically significant changes in forward locomotion, whereas peripheral locomotion was statistically significantly decreased at the 0.21 mg/kg dose (data not shown).

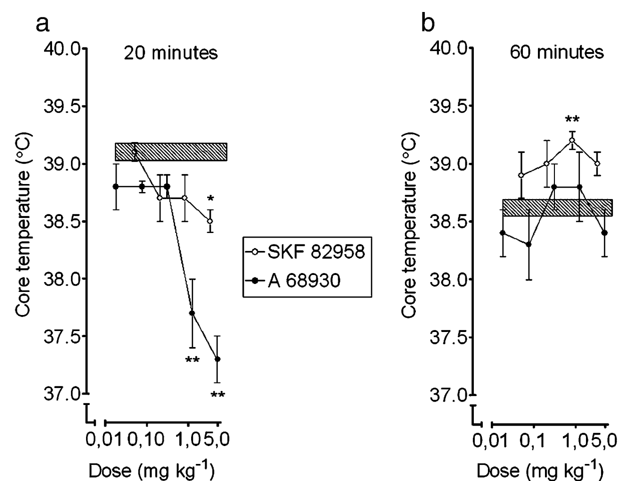

Fig. 2. Effects of A 68930 and SKF 82958 on core temperature. Rats were injected with A 68930 or SKF 82958 and core temperature readings were made at 20 and 60 min after injection. Results are presented as means ± S.E.M., based on observations of 4–6 rats per group. The pooled vehicle-treated controls ($n = 15$) are shown by the shaded area. A 68930 (0.019–4.9 mg/kg) resulted in a marked, dose-dependent and statistically significant decrease in core temperature. SKF 82958 (0.051–3.3 mg/kg) also produced a dose-dependent and statistically significant hypothermia, but of much smaller magnitude (a). Sixty minutes after injection of SKF 82958 there was evidence for hyperthermia (b). This hyperthermic effect was maximal, and statistically significant, at the 0.82 mg/kg dose.

3.2. Effects of A 68930 and SKF 82958 on core temperature

As shown in Fig. 2, the administration of A 68930 (0.019–4.9 mg/kg) resulted in a marked, dose-dependent, and statistically significant, decrease in core temperature. The effect was maximal 20 min after injection and was gone by 60 min. At the 20 min time point, SKF 82958 (0.051–3.3 mg/kg) also produced a dose-dependent and statistically significant hypothermia, but of much smaller magnitude than seen after A 68930. In contrast to the hypothermia produced 20 min after injection of SKF 82958, there was evidence for hyperthermia when the animals were observed 60 min after injection. This hyperthermic effect was maximal, and statistically significant, at the 0.82 mg/kg dose.

3.3. Effects of A 68930 and SKF 82958 on IEG expression

In order to map changes in neural activity after treatment with either of the two agonists we performed in situ hybridizations with oligonucleotide probes against the IEGs c-*fos* and NGFI-A. The rationale for using both those IEGs as markers for neural activity lies in the fact that they show differences in basal expression levels: NGFI-A has a rather high basal expression that makes it possible to detect both reductions and increases in expression (Svenningsson, 1998). The dose (1.6 mg/kg) was based on results from locomotion and core temperature measurements. As shown in Fig. 3c there was a significant increase in expression of c-*fos* mRNA in CPu after A 68930 ($p < 0.01$, one-way ANOVA) and SKF 82958 treatment ($p < 0.001$, one-way ANOVA) with a significantly greater induction of c-*fos* after treatment with SKF 82958 than after treatment with A 68930 ($p < 0.01$, Bonferroni's Multiple Comparison Test). A similar difference in expression was seen for NGFI-A mRNA with a tendency towards a greater induction after SKF 82958 treatment, but here the difference between the two agonist treatments did not reach significance. Results were similar in the rostral and caudal parts of CPu.

In contrast to what was seen in CPu, the two agonists showed marked differences in their ability to induce IEG expression in the accumbens nuclei. No effect was seen on either of the two studied IEGs after treatment with A 68930 (for data on c-*fos*, see Fig. 3c and d, NGFI-A not shown) whereas treatment with SKF 82958 led to a dramatic increase in the expression of both c-*fos* and NGFI-A mRNA. The IEG

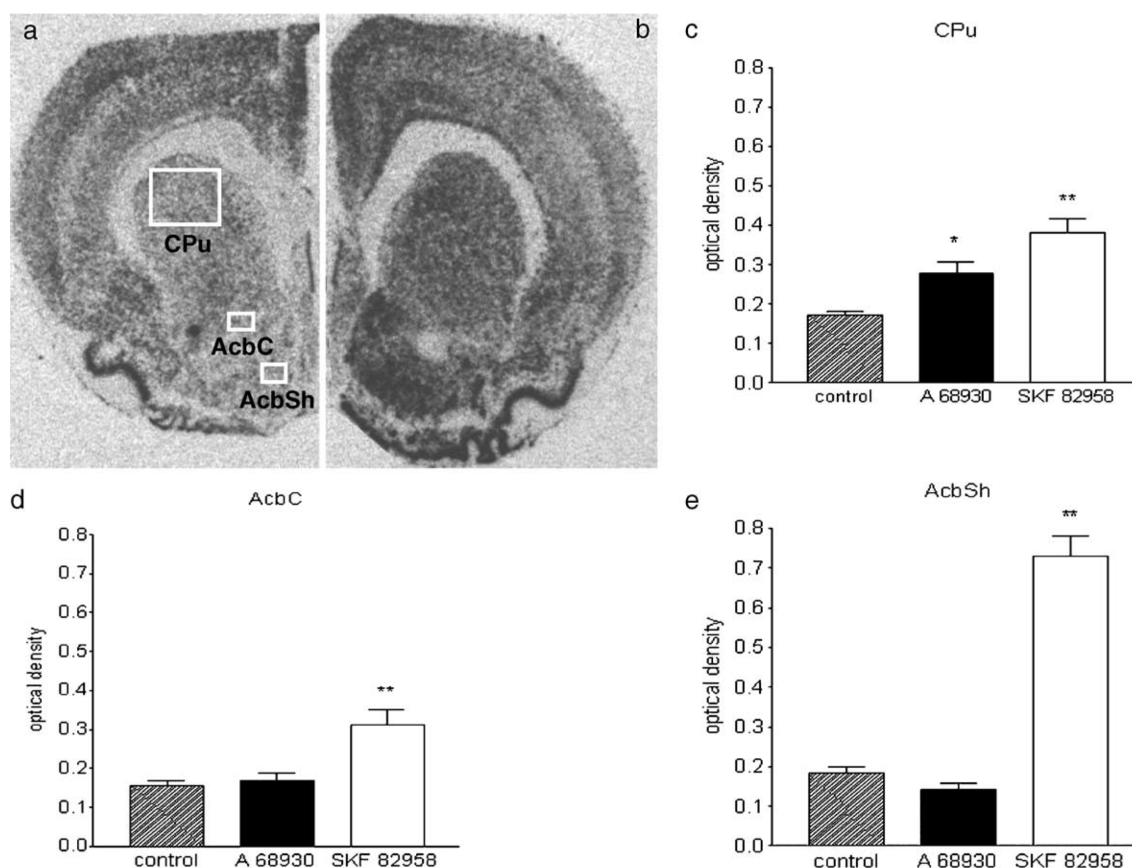

Fig. 3. Effects of A 68930 and SKF 82958 on c-*fos* expression in caudate putamen and the accumbens nucleus. A typical c-*fos* mRNA autoradiogram is shown, saline treated control in panel (a) and after agonist treatment in panel (b). Areas measured are marked with white boxes. Each group contains 6 rats. There was a significant increase in expression of c-*fos* mRNA in CPu (b) after A 68930 ($p < 0.01$, one-way ANOVA) and SKF 82958 treatment ($p < 0.001$, one-way ANOVA) with a significantly greater induction of c-*fos* after treatment with SKF 82958 than after treatment with A 68930 ($p < 0.01$, Bonferroni's Multiple Comparison Test). No effect was seen in the accumbens nucleus after treatment with A 68930 (c and d) whereas treatment with SKF 82958 led to a dramatic increase in the expression of c-*fos*. The amplitude of induction was significantly greater ($p < 0.001$, two-way analysis of variance) in the shell part of the accumbens than it was in the core after treatment with SKF 82958.

expression was increased in both the core and the shell part of the accumbens. However, the amplitude of IEG induction was significantly greater ($p<0.001$, two-way analysis of variance) in the shell part of the accumbens than it was in the core after treatment with SKF 82958. These results were the same for both c-*fos* and NGFI-A.

To see if these cell populations differ with regard to IEG expression following treatment with these two agonists, consecutive sections were hybridized to show expression of the IEG NGFI-A and the neuropeptide preproenkephalin (Enk). The rationale for this is that in striatum, GABAergic medium-sized spiny neurons expressing Enk are those that project to substantia nigra pars reticulata and the entopeduncular nucleus via the external part of globus pallidus and subthalamic nucleus (Gerfen and Young, 1988). This subpopulation of medium sized spiny GABAergic neurons has been shown to express functionally important DA D_2 receptors (Le Moine et al., 1990) but not D_1 receptors. DA D_1 receptors, on the other hand, have been shown to be strongly segregated to neurons expressing substance P/dynorphin but not preproenkephalin (Gerfen et al., 1990).

As expected, both agonists induced IEG expression in neurons that did not express Enk (Fig. 4a and b). We could not detect any induction of NGFI-A in the large cholinergic interneurons (Fig. 4c) or in Enk-positive medium-sized spiny neurons.

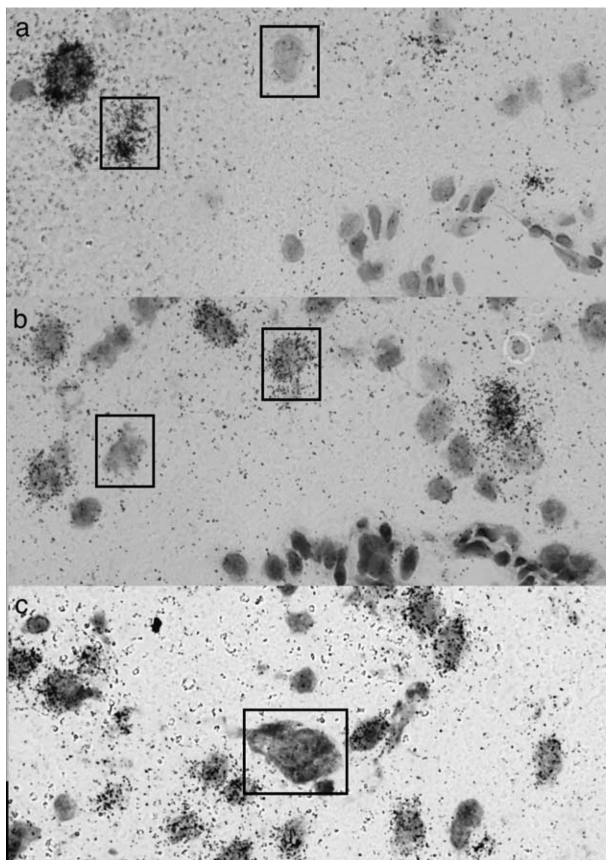

Fig. 4. The cellular distribution of Enk mRNA (a) and NGFI-A mRNA (b) after treatment with SKF 82958. Black boxes indicate an Enk negative, NGFI-A positive and an ENK-positive NGFI-A negative medium sized spiny neuron. (c) shows a large unlabeled cholinergic interneuron.

3.4. Receptor autoradiography

To check for any possible region-specific differences in the binding characteristics of the two agonists, we performed competitive binding against [^{3}H]SCH 23390 with both of the agonists in cortex, dentate gyrus (DG), caudate putamen (CPu), and the accumbens nucleus, shell part (AcbSh) (Fig. 5). Since SCH 23390 binds to both D_1 and D_5 receptors and since some of the binding is still there in D_1 null mutant mice (Montague et al., 2001) the idea was to compare competitive binding curves from regions relatively rich in D_5 receptors, e.g. cortex and hippocampus, and regions relatively poor in D_5 receptors, e.g. CPu (Ariano et al., 1997). We compared the fit of a one-site competition curve and a two-site competition curve with an *F*-test, but found no evidence for more than one binding site for either of the two agonists in any region measured. The EC_{50} values are given in Table 1. The largest difference between the two agonists' ability to displace SCH 23390 binding was that A 68930 was 13 times more potent than SKF 82958 in DG and 12 times more potent in cortex. The smallest difference was noted in CPu, where A 68930 was 9 times more potent than SKF 82958 in displacing SCH 23390.

3.5. Functional assay

Binding experiments cannot tell us if the ligand is an agonist or an antagonist. Given that some members of the class of benzazepine D_1 ligands have an unusual profile (Waddington et al., 2001) it was important to ascertain that SKF 82958 is indeed a full agonist. Therefore, we examined if the two studied agonists, i.e., A 68930 and SKF 82958, have different functional agonist profiles on the receptors within the D_1 family. We used Chinese hamster ovary cells (CHO cells) transfected with either the human D_1 or the human D_5 receptor (Fig. 6a and b). All results are related to dopamine for comparison between assays (Table 2). SKF 82958 had a profile more similar to that of dopamine than did A 68930, with a ratio between the EC_{50} value for SKF 82958 and that of DA of 14 on the D_1 receptors and a ratio of 5 on the D_5 receptor. In contrast, A 68930 was only half as potent as dopamine in stimulating cAMP formation in CHO cells transfected with the D_1 receptor, with an EC_{50} of 0.15 μM compared to 0.07 μM for DA, but approximately 36 times less potent in CHO cells transfected with the D_5 receptor, with an EC_{50} of 0.25 μM for A 68930 and 0.007 μM for DA (Table 2).

4. Discussion

We have confirmed the previous finding that D_1 agonists of the isochroman (e.g. A 68930) and benzazepine (e.g. SKF 82958) groups are two full D1 receptor agonists as regard to cAMP formation but show distinctly different effects on behavior (Waddington et al., 1995). We have extended those observations by demonstrating that they also differ with

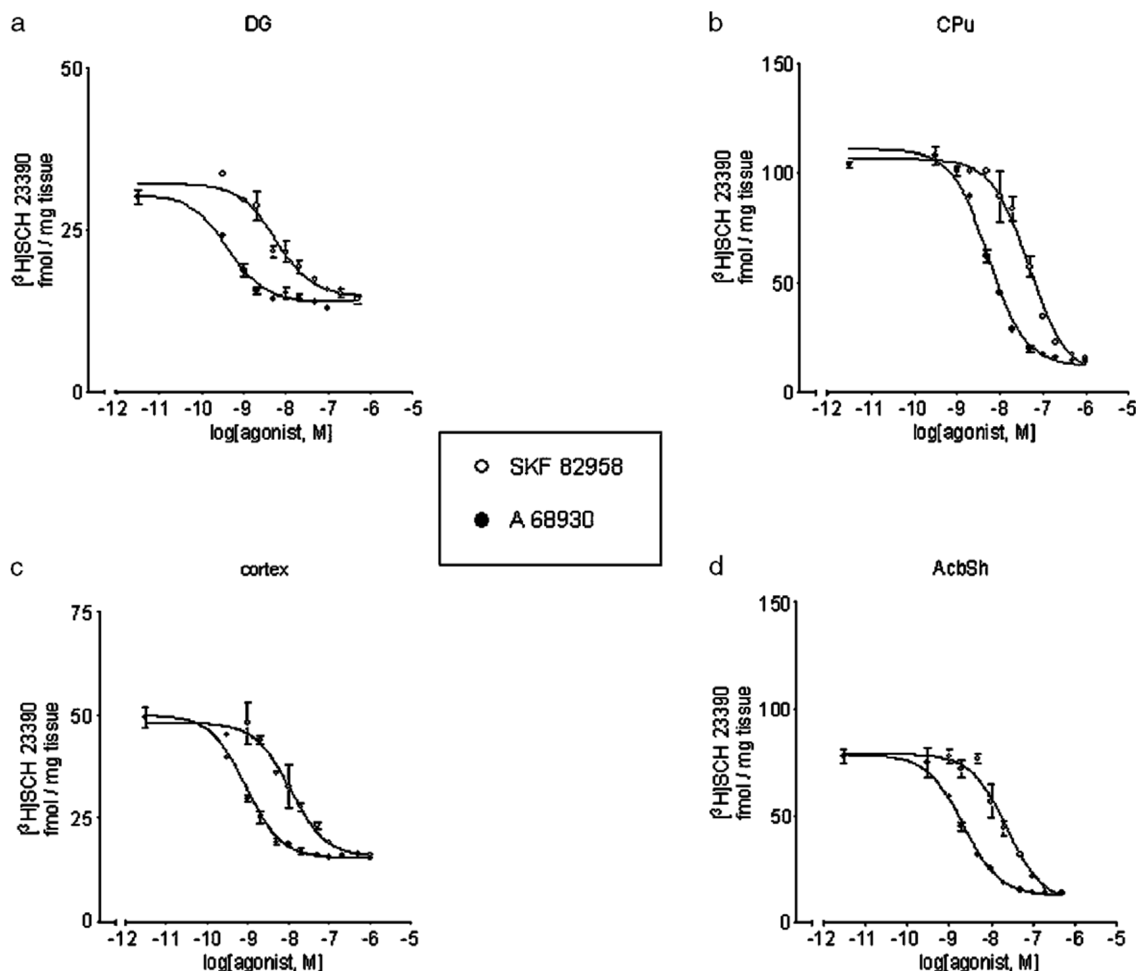

Fig. 5. Competitive binding curves showing [^{3}H]SCH 23390 binding in different brain regions (a to d) in the presence of increasing concentrations of A 68930 or SKF 82958. Antagonist binding is given as fmol bound drug/mg tissue. Corresponding EC_{50} values with 95% confidence intervals are given in Table 1.

regard to the pattern and magnitude of IEG expression they produce and in their ability to alter body temperature. Although there are only subtle differences in the ability of A 68930 and SKF 82958 to displace SCH 23390 in different areas of the brain, there were clear differences in their ability to induce cAMP formation in CHO cells transfected with D_1 and D_5 receptors, respectively. For convenience our results are summarized in Table 3. We will discuss these different aspects below.

Measuring locomotion of rats with equipment that automatically records the breaking of photobeams will obviously pick up a wide variety of behaviors affected by dopamine (Waddington et al., 1995). We are aware that the technique does not discriminate between all these components and that subtle differences may be missed. However, we find clear differences between the two D_1 specific agonists even with this relatively low resolution technique. Thus, in this paper we show that in naïve rats, i.e., rats not given the opportunity to habituate to the open-field arena, A 68930 suppressed locomotion, whereas SKF 82958 had no effect. In contrast to this, A 68930 had no effect and SKF 82958 stimulated spontaneous locomotor activity in rats habituated to the open-field arena. The locomotor activity in the habituated animals is elevated after administration of SKF 82958 to the same extent as it is in the naïve animals not given any drug.

The differences between naive and habituated mice are partly related to their different level of activation of the dopamine system. Thus, it has been shown that dopamine levels rise in the accumbens nuclei following introduction to a novel environment (Rebec et al., 1997). One possible interpretation of our results is that SKF 82958 activates the same brain regions as endogenous dopamine released when animals are exposed to new surroundings and that it has no effect over and above this. Thus, the effect of SKF 82958 on locomotion is similar to that of dopamine. To test this hypothesis we looked for differences in IEG expression in the accumbens nuclei after SKF 82958 treatment and found a clear cut increase. The previously described increase in dopamine efflux during novelty-exploring behavior (Rebec et al., 1997) is restricted to the shell region of the accumbens and no increase in DA efflux was recorded from the core. This is in agreement with our finding that SKF 82958 treatment gave rise to a much stronger induction of IEG in the shell than in the core of the accumbens.

After injection of A 68930, locomotor activity was reduced or unaffected and IEG expression in the shell region of the

Table 1
EC_{50} for the two D_1 agonists A 68930 and SKF 82958 in different brain regions calculated from competitive binding with [^{3}H]SCH 23390

Agonist	Region	EC_{50} (nM)	
A 68930	DG	0.42	(0.28–0.62)
	CPu	5.51	(4.49–6.76)
	AcbSh	2.18	(1.47–3.23)
	Cortex	0.90	(0.54–1.47)
SKF 82958	DG	5.74	(3.30–9.98)
	CPu	49.1	(34.0–70.8)
	AcbSh	23.9	(14.0–40.9)
	Cortex	10.9	(5.65–21.0)

The 95% confidence intervals are given in brackets. DG — dentate gyrus; CPu — caudate putamen; AcbSh — accumbens nucleus shell.

accumbens was not affected — or even decreased. This would therefore be interpreted to mean that A 68930 does not mimic the ability of dopamine to activate relevant neurons in accumbens. A 68930 decreases locomotion in naïve animals. Following our reasoning above regarding differences in dopamine between naive and habituated animals this suggests that A 68930 somehow alters DA receptor signalling in the accumbens under conditions with high endogenous DA levels. In fact, it has recently been shown that A 68930 can block D-amphetamine induced hyperactivity (Isacson et al., 2004), which is due mainly to such elevation of endogenous dopamine.

Furthermore, our results support the notion that there is a clear relation between neural activity in the accumbens nucleus and locomotion (see Svenningsson et al., 1995), and also with the known role of nucleus accumbens shell in generating locomotion (Kelly and Iversen, 1976). Since the IEG experiments were done to see if there were any differences in forebrain regions activated that could explain the observed differences in motor behavior after treatment with A 68930 or SKF 82958 we choose the agonist dose from the locomotor dose–response curve.

We also find support for the idea that SKF 82958 exerts effects close to DA from the fact that SKF 82958 is the only benzazepine that mimics the stimulatory effect of dopamine on adenylate cyclase from rat striatum (O'Boyle et al., 1989) and that SKF 82958 also shows a discriminative-stimulus profile closer to dopamine (or the functional agonist cocaine) than other full D1 agonists (Desai et al., 2003). The authors explain the lack of SKF 38393-like responses after SKF 82958 treatment with SKF 38393 acting via D_1 receptor coupling to different signaling pathways than cAMP. We propose an alternative explanation. Our data indicate that the differences in discriminative-stimulus effects could be due to differences in brain areas activated by different D_1 agonists and we propose an explanation for this that do not need novel D_1 receptor coupling.

In the present study both A 68930 and SKF 82958 induced expression of the two studied IEGs in the CPu even though there were differences in amplitude, with a stronger induction after SKF 82958 treatment. The increase in IEG expression was found predominantly or exclusively in the striatonigral neurons, i.e., those that express D_1 receptors. This strongly supports the idea that A 68930 and SKF 82958 exert their effects on motor behavior observed in this paper independent of D_2 activation. This does not in any way contradict data that motor behavior activated by a D_1 agonist can be influenced by the level of D_2 activation (see e.g. Makihara et al., 2004). Our IEG data show that different D1 agonists can activate quite different neuronal circuits and do so without activating D2 receptors. This is further supported by the fact that no increase in IEG expression was found in interneurons with large cell bodies, i.e., cholinergic interneurons. Given that D_1 family receptors (e.g. D_5 receptors) are found in those cells (Rivera et al., 2002) it may be surprising that we saw no increase in IEG expression there. However, it has previously been found that IEG expression in those cells increases when D_1/D_5 receptors are stimulated together with D_2 receptors, but not upon D_1/D_5 stimulation alone (Svenningsson et al., 2000), indicating that the effect of both A 68930 and SKF 82958 was DA D_1 family mediated.

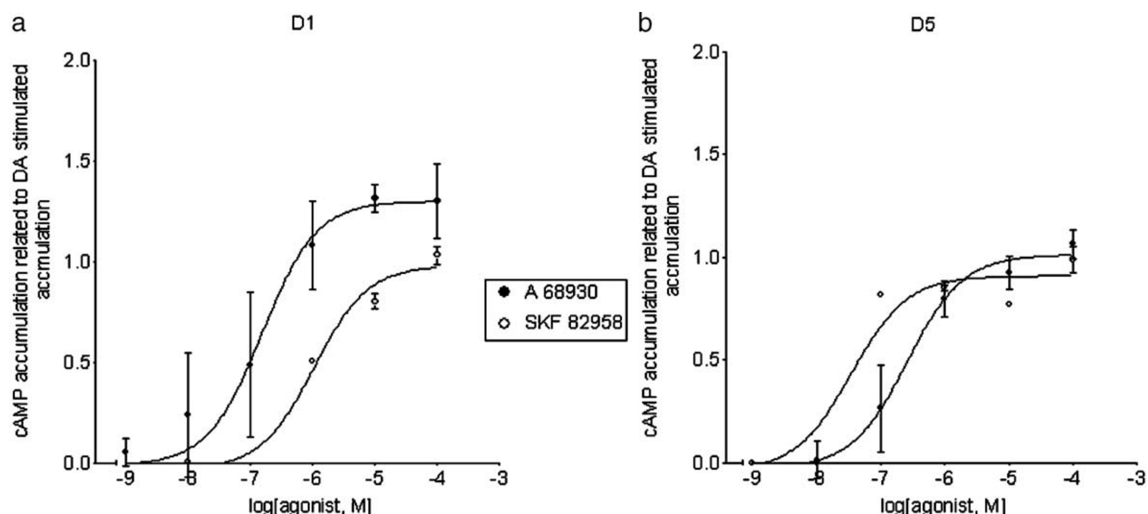

Fig. 6. Effects of A 68930 and SKF 82958 on cAMP accumulation in CHO cells. Values are normalized to the effect of dopamine. EC_{50} values with 95% confidence intervals are given in Table 2.

Table 2
EC_{50} calculated from the two D_1 agonists (A 68930 and SKF 82958) and dopamine's ability to induce cAMP accumulation in CHO cells transfected with D_1 or D_5 receptors

Agonist	Receptor						D_5/D_1
	D_1			D_5			
	EC_{50} (μM)		Relative potency	EC_{50} (μM)		Relative potency	
Dopamine	0.0726	(0.019–0.28)	1.00	0.0069	(0.004–0.010)	1.00	0.1
A 68930	0.1511	(0.047–0.489)	0.5	0.2514	(0.115–0.567)	0.03	1.7
SKF 82958	1.039	(0.723–1.49)	0.07	0.050	(0.018–0.073)	0.2	0.04

95% confidence intervals are given in brackets. For comparison, EC_{50} values are related to the EC_{50} value for dopamine.

Our data show that both D_1 receptors agonists increase activity in striatonigral neurons in the CPu, but that SKF 82958 is considerably more potent. This could suggest that SKF 82958 is a more potent agonist than A 68930 at the relevant D_1 receptor . However, our binding data in CPu and accumbens nucleus suggest the opposite: A 68930 was consistently some 10 times more potent in displacing the DA D_1 family ligand [^{3}H]SCH 23390.

One way to explain the failure of A 68930 to induce locomotion per se and to suppress locomotion induced by a novel environment – presumably mediated via endogenous dopamine – is that it acts as a weak partial agonist. However, both compounds studied were as efficacious as dopamine in the cAMP assay. Instead our data support the suggestion that a major reason for the difference rests in their different abilities to induce activity in neurons of AcbSh.

In the cAMP assay we found that both dopamine and SKF 82958 were at least ten times more potent in activating D_5 than D_1 receptors. By contrast, A 68930 was, if anything, less potent on D_5 than D_1 receptors. Thus, our results would be consistent if dopamine released in vivo during exposure to a novel environment, or SKF 82958 given to habituated animals in our experiment, effectively activated not only D_1 receptors but also D_5 receptors, and that their strong synergistic interaction, especially in the nucleus accumbens shell, would be required in order for a behavioral stimulation to ensue. We find support for this from the full investigation on the behavioral phenotype of the D_5 null mutant mice (Holmes et al., 2001). The authors conclude that the functional relationship between D_1 and D_5 receptors in mediating locomotor activity is likely cooperative.

Table 3
Differences between the two studied D_1 receptor agonists (A 68930 and SKF 82958)

	A 68930	SKF 82958
Locomotion		
Naive	–	0
Habituated	0	+
Temp.	–	+
IEG		
CPu	+	+
AcbSh	0	++
Disp. SCH	+++	++
cAMP form.		
D1	++	+
D5	++	+++

Temp. — core temperature; IEG — expression of immediate early genes; Disp. SCH — displacement of SCH 23390; cAMP form. — formation of cyclic AMP; CPu — caudate putamen; AcbSh — accumbens nucleus shell. 0 indicates no effect, + indicates an effect and +++ indicates a strong effect.

SCH 23390, known to bind with essentially the same affinity to both D_1 and D_5 receptors, was displaced better by A 68930 than by SKF 82958 in all the regions examined. Indeed, A 68930 was approximately 10 times more potent as a displacer and this was true also in regions where D_5 receptors are believed to be relatively abundant such as hippocampus. However, the approximately 10 fold difference in potency as a displacer of SCH 23390 agrees reasonably well with the fact that A 68930 was almost 10 times more potent in the cAMP assay. Thus, our autoradiography data only suggest that throughout the brain SCH 23390 finds many more D_1 than D_5 receptors.

This has important functional consequences as it is known that the potency of an agonist in a functional context depends both on the affinity of the agonist to the receptor and also on the number of receptors present (Arslan et al., 1999; Johansson et al., 2001; Kenakin, 2002). Therefore, in order to activate, at a given dose, both D_1 and D_5 receptors, the agonist must be more potent at D_5 than at D_1 receptors, because the latter are so much more abundant. In our functional assay this means that A 68930 will be an almost pure D_1 agonist, whereas dopamine and SKF 82958 activate both D_1 and D_5 receptors to an approximately equal extent.

This interpretation is also relevant when we examine the effects of the two drugs on core temperature. Here we find that A 68930 is much more potent than SKF 82958, consistent with its higher D_1 potency. Hence, if our interpretation is correct, our data would imply that the acute decrease in core temperature is almost exclusively mediated by D_1 receptors. This could in fact explain why the D_1 agonists, even though they differ in other aspects (e.g. orofacial movements etc.), both decrease core temperature. We propose that D_5 receptors are not involved in body temperature control and that this could provide a way to study pure D_1 effects of D1-agonists not selective for D_1/D_5 receptors. We do not think that the difference in core temperature between the two D1 agonists seen after 60 min is specific enough to add to the discussion about D_1/D_5 activation. Core temperature at this time point is most likely a sum of heat generated from metabolism and physical activity. One possible explanation for the increase in core temperature after the intermediate dose but not after the higher dose of SKF 82958 could be that at the higher dose core

temperature are maximally lowered due to D1 activation (seen at 20 min) and that the sum at 60 min therefore are lower than after the higher dose of SKF 82958.

The functional D_1 selectivity of A 68930 that we propose is, however, critically dependent on a high abundance of D_1 receptors and is by no means absolute. Indeed, in D_1 knock-out mice where there are no D_1 receptors and appropriate adaptive changes have taken place the compound can produce behavioral activation (Clifford et al., 1999; Waddington et al., 2001). Furthermore, it must be emphasized that there is still a possibility that dopamine receptor agonists could act on a receptor importantly different from archetypical D_1 or D_5 receptors (see Waddington et al., 2001).

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