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RESEARCH PAPER

Pharmacological characterization and CNS effects of a novel highly selective α_{2C}-adrenoceptor antagonist JP-1302

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Background and purpose: Pharmacological validation of novel functions for the α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptor (AR) subtypes has been hampered by the limited specificity and subtype-selectivity of available ligands. The current study describes a novel highly selective α_{2C}-adrenoceptor antagonist, JP-1302 (acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine).
Experimental approach: Standard *in vitro* binding and antagonism assays were employed to demonstrate the α_{2C}-AR specificity of JP-1302. In addition, JP-1302 was tested in the forced swimming test (FST) and the prepulse-inhibition of startle reflex (PPI) model because mice with genetically altered α_{2C}-adrenoceptors have previously been shown to exhibit different reactivity in these tests when compared to wild-type controls.
Key results: JP-1302 displayed antagonism potencies (K_B values) of 1,500, 2,200 and 16 nM at the human α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptor subtypes, respectively. JP-1302 produced antidepressant and antipsychotic-like effects, *i.e.* it effectively reduced immobility in the FST and reversed the phencyclidine-induced PPI deficit. Unlike the α_2-subtype non-selective antagonist atipamezole, JP-1302 was not able to antagonize α_2-agonist–induced sedation (measured as inhibition of spontaneous locomotor activity), hypothermia, α_2-agonist-induced mydriasis or inhibition of vas deferens contractions, effects that have been generally attributed to the α_{2A}-adrenoceptor subtype. In contrast to JP-1302, atipamezole did not antagonize the PCP-induced prepulse-inhibition deficit.
Conclusions and implications: The results provide further support for the hypothesis that specific antagonism of the α_{2C}-adrenoceptor may have therapeutic potential as a novel mechanism for the treatment of neuropsychiatric disorders.
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Keywords: α_2-antagonist; α_{2C}-adrenoceptor; prepulse inhibition; forced swimming test

Abbreviations: AR, adrenoceptor; CHO cells, Chinese hamster ovary cells; FST, forced swimming test; JP-1302, acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine; PPI, prepulse inhibition of startle reflex

Introduction

α_2-Adrenoceptors (α_2-ARs) are known to have a critical role in regulating neuronal firing and neurotransmitter release in the central nervous system (CNS) (Ruffolo *et al*., 1993). The α_2-ARs consist of three genetically and functionally distinct subtypes, A, B and C. The rodent orthologue of the human α_{2A}-AR is sometimes called the α_{2D}- or $\alpha_{2A/D}$-AR; here, the term α_{2A}-AR includes both human and rodent α_{2A}-AR, if not further specified. The α_{2A}-AR subtype is the most prominent subtype and is widely distributed throughout the body. In contrast, the expression of the α_{2C}-AR is more limited and concentrated in specific areas of the CNS, with the highest expression levels of α_{2C}-AR being found in the olfactory tubercles, striatum and hippocampus (Scheinin *et al*., 1994; Rosin *et al*., 1996). Therefore, it has been suggested that the α_{2C}-AR may have a special role in the CNS, although it may also be of some significance outside the CNS, for example in the regulation of cardiovascular homeostasis (Sun *et al*., 2001; Brede *et al*., 2004; Regitz-Zagrosek *et al*., 2006). There is evidence from animals with genetically altered α_{2C}-AR expression and models predicting antipsychotic and antidepressant efficacy that α_{2C}-ARs play an important role in the modulation of monoamine neurotransmission in the brain, especially under stressful conditions (MacDonald *et al*., 1997; Scheinin *et al*., 2001). In addition, it has been

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suggested that α_{2C}-AR antagonism may contribute to the therapeutic benefit of certain antipsychotic compounds, such as clozapine (Kalkman and Loetscher, 2003; Arnsten, 2004), but the significance and nature of α_{2C}-antagonism in the actions of currently used antipsychotics is still unclear owing to the fact that these compounds act on multiple pharmacological targets. A recent study has also detected a significant association between α_{2C}-polymorphism and abnormal neuropsychological responsiveness in patients suffering from major depressive disorder (Neumeister *et al*., 2006).

The forced swimming test (FST) is a widely used paradigm in the screening for novel antidepressants in rodents (Petit-Demouliere *et al*., 2005). The genetic deletion of the α_{2C}-AR (α_{2C}-knockout (KO) mice) produced antidepressant-like effects in the FST, whereas the overexpression of the α_{2C}-AR (α_{2C}-OE mice) had the opposite effect (Sallinen *et al*., 1999). The prepulse inhibition of the startle reflex (PPI) is another experimental model relating to the so-called sensorimotor gating phenomenon (Swerdlow *et al*., 1994) that is currently being extensively studied in the preclinical screening of putative antipsychotic-like CNS compounds. In mice, the α_{2C}-KO and α_{2C}-OE mutations were associated with lower and higher levels of PPI, respectively (Sallinen *et al*., 1998). In addition, the α_{2A}-KO mutation has been found to increase PPI levels, suggesting that the α_2-AR subtypes have distinct roles in the modulation of PPI (Lähdesmäki *et al*., 2004). Based on the above studies, it has been suggested that α_{2C}-AR-specific compounds might have therapeutic use in certain CNS disorders, such as depression and schizophrenia.

The analysis of the functional and pharmacological role of the α_{2C}-AR has for a long time been hampered by the lack of agonists and antagonists with sufficient α_2-AR subtype-selectivity (Mayer and Imbert, 2001). The need to use transgenic mouse models may lead to erroneous interpretations when results are extrapolated to predict the actions of specific agonists or antagonists in non-genetically modified conditions. It is clearly important to test hypotheses originating from results with transgenic animals and ligands of limited specificity with more subtype selective ligands when they become available. Therefore, the highly α_{2C}-selective and specific ligand JP-1302 (acridin-9-yl-(4-(4-methylpiperazin-1-yl)-phenyl)amine) was characterized here for its primary pharmacological properties and tested for its CNS efficacy in the FST and PPI paradigms, assays in which α_{2C}-AR-selective ligands have been predicted to have therapeutic effects (Scheinin *et al*., 2001). The results suggest that JP-1302 is a suitable experimental tool to study α_{2C}-adrenoceptor–mediated functions *in vivo*.

Methods

Animals

Unless otherwise stated, adult male 2–4-month-old Spraque–Dawley (SD) rats or NMRI mice, purchased from Scanbur B&K (Sollentuna, Sweden) were used as the source of tissue preparations and as the subjects of the *in vivo* experiments. Rats were housed in groups of five animals under standard conditions with lights on from 0600 to 1800 h, the room being dark during the remaining 12 h. The room temperature was kept at 20–22°C and the relative humidity at 45–65%. The animals received standard pellets (Special Diet Service, Essex, England) and tap water *ad libitum*. The animals were acclimatized to the test laboratory at least 1 week before experiments. Behavioural studies were conducted between 0800 and 1600 h and the animals were transferred to the study room at least 1 h before the start of the experiment. The animal phase of the study was performed according to the rules of the Council of Europe and the National Research Council of USA, and the study was approved by the Provincial State Office of Western Finland.

In vitro *studies*

Radioligand-binding assays. The affinity of test compounds for the three human α_2-adrenoceptor subtypes (α_{2A}, α_{2B} and α_{2C}) and the mouse α_{2D} subtype was determined in competition-binding assays with [^{3}H]-rauwolscine and [^{3}H]-RX821002, respectively. The biological material in the [^{3}H]-rauwolscine displacement assay consisted of membranes from Shionogi S115 cells stably transfected with one of the three human α_2 subtypes (Marjamäki *et al*., 1992). In the [^{3}H]-RX821002 displacement assay, membranes from Chinese hamster ovary (CHO) cells stably transfected with the mouse α_{2D} subtype were used. The membrane suspensions (3–15 μg total protein per sample, depending on the expression level of individual subtypes) and about 1 nM of [^{3}H]-rauwolscine (specific activity 75–85 Ci mmol^{-1}) or [^{3}H]-RX821002 (specific activity 59 Ci mmol^{-1}) were incubated with a minimum of six concentrations of the test compound in a total volume of 90 μl (50 mM KH_2PO_4, pH 7.5, at room temperature). Specific-binding was defined by 100 μM oxymetazoline and corresponded to 90–96% of the total binding. After 30 min at room temperature, the incubations were terminated by rapid filtration (TomTec 96 harvester. Tomtec Inc, Hamden, CT, USA) through presoaked GF/B glass-fibre mats (Wallac Oy, Turku, Finland) and three washes with ice-cold 50 mM KH_2PO_4 (pH 7.5 at room temperature). The filter mats were then dried and a solid scintillate (Meltilex; Wallac Oy) was melted onto them before their radioactivity was measured (BetaPlate; Wallac Oy).

The analysis of competition binding experiments was carried out by nonlinear least square curve fitting. IC_{50}s were converted to K_i values by using the equation of Cheng–Prussoff ($K_i = IC_{50}/(1 + ([^3\text{H-ligand}]/K_{d,\ 3H-ligand})$).

The affinity profiling of JP-1302 at a concentration of 0.1 and 10 μM on a number of receptors other than the α_2-adrenoceptor subtypes was conducted by Cerep (Le Bois Lévêque, Celle L'Evescault, France) using documented standard procedures (see Table 2 for relevant details of the experimental conditions).

α_2-Antagonist activity in cellular membranes

The antagonist activity of JP-1302 was determined as the ability of the compound to inhibit adrenaline-stimulated ^{35}S-guanosine-5′-*O*-(3-thio)triphosphate (^{35}S-GTPγS)-binding to G-proteins competitively (Jasper *et al*., 1998) in mem-

branes of CHO cells stably transfected with one of the three human α_2 subtypes (Pohjanoksa *et al*., 1997). Membranes (2–6 μg of protein per sample) and 12 concentrations of JP-1302 were preincubated for 30 min at room temperature in 50 mM Tris, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 10 μM GDP, 30 μM ascorbic acid, pH 7.4, with a fixed concentration of adrenaline (5 μM for α_{2A}, 15 μM for α_{2B}, 5 μM for α_{2C}). Then trace amounts of [^{35}S]-GTPγS (0.08–0.15 nM, specific activity 1250 Ci mmol^{-1}) were added to the incubation mixture. After an additional 30 min at room temperature, the incubation was terminated by rapid vacuum filtration through glass fibre filter. Filters were washed three times with 5 ml ice-cold wash buffer (20 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4), dried and counted for radioactivity in a scintillation counter. Experiments were repeated at least three times.

The analysis of antagonism experiments was carried out by nonlinear least-square curve fitting. IC_{50}s were converted to K_B values by using the equation $K_B = IC_{50}/(1 + (\text{adrenaline})/EC_{50,\,\text{adrenaline}})$ with EC_{50} values of adrenaline on the three α_2-AR subtypes of 0.76 μM (α_{2A}), 2.4 μM (α_{2B}) and 0.71 μM (α_{2C}).

Antagonist activity in the vas deferens model

Studies were performed by using a four-position Schuler organ bath with a tissue chamber volume of 10 ml (Hugo Sachs Elektronik, March-Hugstetten, Germany). The temperature of the chambers was kept constant (37°C) with a circulated water bath (Hetofrig, Heto, Birkerød, Denmark). Isometric contractions of the preparations were measured by a Grass force–displacement transducers model FT03 (Grass Instruments, Quincy, MA, USA) connected to Grass DC low-level pre-amplifiers and Grass driver amplifiers type 7DAG. The amplified signals were plotted onto paper by a Grass ink writer oscillograph type 7WU 16F. Electrical stimulation pulses were delivered by a Grass two channels constant voltage stimulator type S88. For the field stimulation of vas deferens preparations, tissue holder type CR Pt with field stimulation electrodes (Hugo Sachs Elektronik) were used.

The buffer consisted of Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1. The buffer was gassed at a temperature of 37°C with 5% carbogen for at least 30 min before the pH was adjusted to 7.4. Propranolol (260 μg l^{-1}) and desipramine (2 μg l^{-1}) were added to the solution to prevent possible effects of test compounds on β-adrenoceptors and the re-uptake of released noradrenaline, respectively.

Rats were killed by CO_2-suffocation. Vasa deferentia were dissected out and the prostatic halves were transferred to tissue chambers containing Krebs solution. Preparations were tied to the bottom hooks of the incubation chambers and the isometric force–displacement transducers and allowed to stabilize under a resting tension of 0.5*g* for 5–10 min. Then the electrical stimulation of prostatic vas deferens segments was started by applying field stimulations with the following parameters: twin-pulses, delay 5 ms, frequency 0.2 Hz, voltage 70 V, duration 2 ms. The isometric contractions of the segments in response to the electrical stimulations were recorded and allowed to stabilize before establishing the dose–response curve for the agonist dexmedetomidine. For this purpose dexmedetomidine was administered cumulatively with half-logarithmic increments at 2 min intervals. JP-1302 or atipamezole were added 5 min before the first agonist dosing in a volume of 80 μl. In control preparations, an equal volume of the vehicle was added. For JP-1302, 100% dimethylsulphoxide (DMSO) was used as vehicle.

The inhibition of electrically evoked contractions by dexmedetomidine was considered as an indication of agonism at α_2-ARs and agonist effects were expressed as per cent inhibition of the electrically evoked contractions. Means and standard deviation (s.d.) of replicates were calculated and used to construct agonist dose–response curves in the absence and presence of antagonists. pD_2-values for dexmedetomidine were determined from sigmoidal dose–response curves by employing the following equation: $Y = \text{Bottom} + ((100 - \text{Bottom})/(1 + 10(\log EC_{50} - X)))$ (GraphPad Prisma 3.0, GraphPad Software Inc., San Diego, CA, USA), where Y is the response, % inhibition; X the log(antagonist), Bottom the lowest response = 0%. pA_2-values for antagonists was calculated with the following formula: log(DR 1) log A, where DR is the dose ratio for agonist with and without antagonist and log A is the logarithm of the corresponding antagonist dose. The averages of two pA_2-values at two different antagonist concentrations (10 and 100 nM for atipamezole and 10 μM for JP-1302) were calculated and used for final results.

In vivo *studies*

Antagonism of the mydriatic effect of the α_2-agonist dexmedetomidine. Rats ($n = 5$/group, total of 15) were anaesthetized with sodium pentobarbitone (Mebunat 60 mg ml^{-1}, Orion, Finland) and a polyethylene cannula was inserted into the lateral tail vein for drug administration. The pupil diameter was measured by means of an operating microscope provided with a 10-mm graduated line (0.1 divisions) in the ocular. The microscope had an internal light source with green filter. The light was maintained at a steady intensity throughout the experiments. After measurement of the baseline pupil diameter, all rats were given the α_2-agonist dexmedetomidine 10 μg intravenously (i.v.). The mydriatic effect of dexmedetomidine was measured after 5 min and then cumulative doses of either atipamezole or JP-1302 or equivalent volumes of vehicle were applied intravenously at 5 min intervals. The cumulative doses of the antagonists were determined with steps of 3, 10, 30, 100, 300, 1000 and 3000 nmol kg^{-1}, and the entire duration of the measurement was thus 45 min. The pupil diameter measurements were performed just before the next injection.

Antagonism of α_2-agonist–induced inhibition of locomotor activity (sedation) and hypothermia. Spontaneous locomotor activity of a total of 76 male NMRI mice (B&K, Sweden) was measured by placing individual animals into a polypropylene animal cage ($38 \times 22 \times 15$ cm). The cages were surrounded by an infrared photobeam frame system designed for activity measurements (Photobeam Activity System PAS, Cage Rack, San Diego Instruments, San Diego,

CA, USA). The animals were injected either with JP-1302 or atipamezole 20 min before the injection of dexmedetomidine (50 nmol kg^{-1} s.c.). Spontaneous locomotor activity was measured 20 min after dexmedetomidine injection and the dexmedetomidine–induced inhibition of locomotor activity was used as a measure of sedation. At the end of the locomotor activity recordings, the core body temperatures of the mice were measured with a rectal probe and a digital thermometer (Ellab, Roedovre, Denmark). The probe was inserted 2.5 cm inside the anal sphincter and maintained there until the temperature reading of the thermometer was stabilized. The same system was used in a separate experiment for the detection of the effects of JP-1302 alone. In the latter experiment, the activity was recorded for the period of 20–40 min after the drug injection.

Effect of JP-1302 in the FST for antidepressant activity

Rats were transferred into the experimental room at least 30 min before testing. Forced swimming was conducted by immersing each rat individually in a transparent glass cylinder (height 46 cm, diameter 20 cm) containing a 21-cm deep column of water at 25°C. In the FST sessions, an initial 15 min pre-test was followed 24 h later by a 5-min actual test. Drug treatments, as two subcutaneous (s.c) injections, were given during the period between the two sessions, the first 15 min after the pre-test and the second 1 h before the test swim. Following both FST sessions, the rats were removed from the cylinders, dried with towels and placed into heated cages for 15 min, and then returned to their home cages. Each animal was used only once.

The cumulative time of immobility was directly observed and recorded by a stopwatch timer during the 5 min test swim. The experimenter was well experienced in rating the behaviour and blinded for the different drug treatments. A rat was judged to be immobile when it remained floating in the water without struggling and was making only those movements necessary to keep its head above water.

Effect of JP-1302 and atipamezole on prepulse inhibition of acoustic startle

Startle experiments were performed in four identical, ventilated and illuminated startle chambers ($39 \times 38 \times 58$ cm (length $\times$ width $\times$ height)) (SR-LAB System, San Diego Instruments). The chambers consisted of a non-restrictive Plexiglas cylinder (3.9 cm in diameter) resting on a Plexiglas platform. Piezoelectric accelerometers mounted under the cylinders detected and transduced the animal movements. High-frequency speakers (Radio Shack Supertweeter, San Diego, CA, USA), mounted 25 cm above the cylinder, provided all acoustic stimuli. Presentation of the acoustic stimuli and the piezoelectric responses from the accelerometer were controlled and digitized by the SR-LAB software and interface system. The sensitivity of the chambers was adjusted to average readings of 100 using the standardization unit from San Diego Instruments. Sound levels within each chamber were measured repeatedly using the A weighing scale (Radio Shack Sound Level Meter, Fort Worth, TX, USA) and were found to remain constant. As differences in PPI levels between different rat strains can contribute to drug responses, two rat strains were used (SD and Wistar) to test the effect of JP-1302.

The following session protocol was employed in all experiments: after a 5-min habituation period, rats were exposed to 30 PULSE ALONE trials and three types of PREPULSE + PULSE trials, consisting of eight trials each (total 24 PREPULSE + PULSE trials). In these three different types of PREPULSE + PULSE trials, the prepulse-intensity varied and was 3, 6 or 15 dB above the 72 dB background noise level. Programmed file names of these PREPULSE + PULSE trials were PRE3, PRE6 and PRE15, respectively, and the PULSE ALONE trial name was PULSE. The PULSE intensity was 118 dB and the duration of both PULSE and PREPULSE was 40 ms. PREPULSE–PULSE interval was 100 ms. The total duration of a startle session was 21 min (including habituation). On average, there was a 13-s interval (range 5–30 s) between trials. Trials were administered in a pseudo-random order.

The extent of PPI, that is, the prepulse inhibition percentage after various prepulse intensities, was calculated using the averaged individual values from the different trials according to the formula [[average PULSE – average (PRE3 or PRE6 or PRE15)]/average PULSE]*100.

Data analysis and statistical procedures

SPSS 12.0.1. for Windows software package (Chicago, IL, USA) was used for the statistical analysis of the experimental data. Repeated measurements analysis of variance (ANOVA) was used in the analysis of the mydriasis model data. One-way analysis of variance followed by the least-significant difference (LSD) test was used to compare the differences of drug treatment groups in the spontaneous locomotor activity measurements and in the FST. The nonparametric Kruskall–Wallis ANOVA and Mann–Whitney *U*-tests were used for the startle reflex and PPI results owing to the lack of normal distribution of these data. *P*-values < 0.05 were considered statistically significant.

Drugs

JP-1302 (acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl] amine) was discovered through a high-throughput screening campaign aimed at identifying α_{2C}-selective compounds. The compound was synthesized in Juvantia Pharma Ltd (Turku, Finland) and by Pharmatory Ltd (Oulu, Finland). The synthesis has been described in a patent application of Orion Corporation and Juvantia Pharma by Wurster *et al*. (2001), PCT no. WO01/64645 A2. The structural formula is shown in Figure 1. A hydrochloride salt form of JP-1302 was used for *in vivo* experiments. Dexmedetomidine HCl, atipamezole HCl, propranolol HCl and fluoxetine HCl were synthesized by Orion Corporation (Espoo, Finland). Desipramine HCl and phencyclidine (PCP) HCl were purchased from Sigma (St Louis, MO, USA). All compounds were dissolved either in dimethylsulphoxide (DMSO) (*in vitro* studies) or in distilled sterile water and/or in physiological salt solution (*in vivo* studies). In order to increase solubility, the pH was acidified by 0.1 M HCl for JP-1302. The formulations for all drug

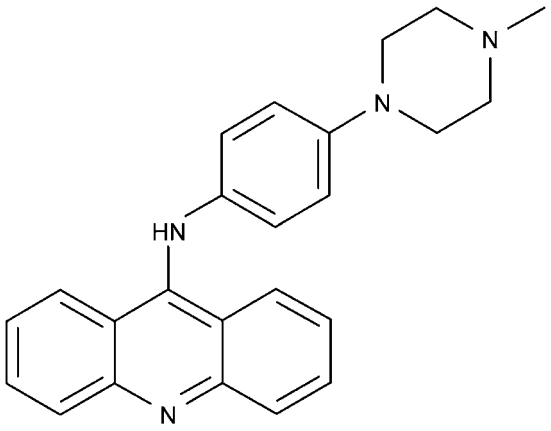

Figure 1 The chemical structure of JP-1302. Molecular weight is 368.47.

injections are expressed here in mol kg^{-1} basis and the injection volume was 1 ml kg^{-1} for rats and 5 ml kg^{-1} for mice. The administration route was i.v. in the mydriasis test and s.c. in all other tests.

Results

In vitro *studies*

Radioligand-binding assays. In competition binding assays with [^{3}H]-rauwolscine, JP-1302 displayed an affinity of 28 nM for the α_{2C}-AR (Table 1). As the affinity of JP-1302 on the three other α_2-AR subtypes were 1500 nM or lower, the compound is endowed with a minimum selectivity of about 50-fold for the α_{2C}-AR. The profiling of JP-1302 at a concentration of 0.1 μM against 30 other receptor targets revealed no discernible secondary sites (Table 2). At a 100-fold higher concentration (10 μM) binding to α_1-ARs and some other receptors was found (Table 2).

Preliminary pharmacokinetic results indicated that with the doses used in the FST and PPI experiments (1–10 μmol kg^{-1}), the C_{max} concentrations in plasma did not exceed 0.3 μM.

α_2-Antagonist activity in cellular membranes. JP-1302 was unable to increase [^{35}S]-GTPγS-binding to membranes of CHO cells expressing the three human α_2-AR subtypes, thereby demonstrating that the compound does not possess agonist activity on α_2-AR (Table 1). JP-1302 was, however, able to antagonize the agonist response of a fixed amount of adrenaline in a concentration-dependent manner (Figure 2). The apparent antagonist potencies (K_B values) of JP-1302 were found to be 1500, 2200 and 16 nM for the α_{2A}-, α_{2B}- and α_{2C}-AR subtypes, respectively (Table 1).

α2-Antagonist activity in the vas deferens model. Dexmedetomidine dose-dependently inhibited electrically evoked contractions in rat vas deferens preparations resulting in EC_{50} values of 1.4 nM (Figure 3). JP-1302 had no antagonist effects on the dexmedetomidine-induced inhibition. However, in the presence of atipamezole, the dose–response curve of dexmedetomidine was shifted rightwards, resulting in pA_2 values for atipamezole of 8.5.

Table 1 *In vitro* binding affinity and functional antagonism values of JP-1302 on human and rodent (α_{2D}) α_2-adrenoceptors

Receptor	*Binding affinity (Ki, nM)*	*Antagonist activity (Kb, nM)*	*Agonistic activity*
α_{2A}	3150 ± 50	1495 ± 270	No agonism
α_{2B}	1470 ± 130	2175 ± 345	No agonism
α_{2C}	28 ± 2	16 ± 6	No agonism
α_{2D}	1700 ± 200	Not determined	Not determined

Abbreviation: JP-1302, acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine. Affinities were determined in competition binding assays using [^{3}H]-rauwolscine or [^{3}H]-RX821002 with membranes from stably transfected cell lines separately expressing one of the subtypes (α_{2A}, α_{2B} and α_{2C}: S115 cells, [^{3}H]-rauwolscine; α_{2D}: CHO cells, [^{3}H]-RX821002). Antagonist potencies were established with membranes from CHO cells stably expressing the human subtypes by antagonizing the adrenaline-induced stimulation of [^{35}S]-GTPγS binding. Numerical results are based on a minimum of three repeat experiments and are expressed as mean ± s.e.m. in nM.

In vivo *studies*

Effects of JP-1302 on baseline behaviour. As shown in Table 3, the administration of JP-1302 did not affect the baseline spontaneous locomotor activity of mice at dose levels that were used in the other *in vivo* experiments (3–30 μmol kg^{-1}); 100 μmol kg^{-1} produced minor and 300 μmol kg^{-1} clear inhibition of locomotor activity. In addition, a slight strengthening of PPI in rats without PCP administrations was observed after 30 μmol kg^{-1}, but not with lower doses (3 or 10 μmol kg^{-1}).

Antagonism of the mydriatic effect of an α_2-agonist. Dexmedetomidine produced a clear mydriatic effect (i.e. pupil dilatation) that was readily antagonized by atipamezole but not by JP-1302 (Figure 4). The mean ± s.e.m. pupil diameter of rats was 0.28 ± 0.02 mm before and 3.95 ± 0.18 mm after dexmedetomidine. A time course effect suggesting the tapering of the agonist effect was seen in the two last measurements, where a statistically significant decrease in pupil diameter was observed compared to the initial value after dexmedetomidine and vehicle ($P<0.004$). However, a clear significant treatment group × time course interaction was detected in pupil diameter between vehicle and atipamezole after the dose of 30 nmol kg^{-1} i.v. ($P=0.031$) as well as after all the higher doses studied ($P<0.001$). Administration of JP-1302 did not produce a statistically significant effect after any dose.

Antagonism of α_2-agonist-induced sedation and hypothermia. Dexmedetomidine (50 nmol kg^{-1} s.c.)-induced profound locomotor inhibition and hypothermia in rats. The reduction in rectal temperature measured 40 min after vehicle + dexmedetomidine treatment was 5.0 ± 0.2°C (mean ± s.e.mean) and the locomotion counts were reduced by 94% compared to vehicle + vehicle-treated group. These effects of dexmedetomidine were potently and dose-dependently antagonized by atipamezole but not by JP-1302

Table 2 Specific radioligand binding values of JP-1302 (% inhibition) at two different concentrations (0.1 and 10 μM) to the receptor studied and IC_{50} values for the reference compounds

Receptors	*Inhibition % 0.1 μM*	*Inhibition % 10 μM*	*Tissue*	*Reference compound*	*Reference compound IC_{50} (nM)*	*Radioligand*	*Radioligand concentration*	*Nonspecific*	*Incubation parameters*
A1	—	—	Rat cerebral cortex	CPA	0.88	[^{3}H]CCPA	0.5 nM	CPA (10 μM)	120 min/22°C
alpha1A	—	93	Rat salivary glands	WB 4101	0.6	[^{3}H]prazosin	60 pM	phentolamine (10 μM)	60 min/22°C
alpha1B	—	97	Rat liver	Spiperone	3	[^{3}H]prazosin	50 pM	phentolamine (10 μM)	60 min/22°C
NE uptake	—	75	Rat cerebral cortex	Protriptyline	2.8	[^{3}H]nisoxetine	1 nM	desipramine (1 μM)	240 min/4°C
BZD (central)	—	33	Rat cerebral cortex	Diazepam	11	[^{3}H]flunitrazepam	0.4 nM	diazepam (3 μM)	60 min/4°C
D1 (human)	—	52	Human recombinant (L cells)	SCH 23390	1	[^{3}H]SCH 23390	0.3 nM	SCH 23390 (1 μM)	60 min/22°C
D2 (human)	—	14	Human recombinant (A9L cells)	(+)butaclamol	8	[^{3}H]spiperone	0.3 nM	(+)butaclamol (10 μM)	60 min/22°C
D3 (human)	—	22	Human recombinant (CHO cells)	(+)butaclamol	12	[^{3}H]spiperone	0.3 nM	(+)butaclamol (10 μM)	60 min/22°C
D4 (human)	—	14	Human recombinant (CHO cells)	Clozapine	100	[^{3}H]spiperone	0.3 nM	(+)butaclamol (10 μM)	60 min/22°C
D5 (human)	—	35	Human recombinant (GH4 cells)	SCH 23390	0.79	[^{3}H]SCH 23390	0.3 nM	SCH 23390 (10 μM)	60 min/22°C
DA uptake	20	85	Rat striatum	Nomifensine	109	[^{3}H]BTCP	0.5 nM	BTCP (10 μM)	90 min/4°C
GABA A	—	43	Rat cerebral cortex	Muscimol	16	[^{3}H]muscimol	5 nM	muscimol (10 μM)	10 min/4°C
GABA B	18	21	Rat cerebral cortex	Baclofen	164	[^{3}H]GABA (+40 μM isoguvacine)	10 nM	baclofen (100 μM)	10 min/22°C
AMPA	—	—	Rat cerebral cortex	L-glutamate	1,090	[^{3}H]AMPA	8 nM	L-glutamate (1 mM)	60 min/4°C
NMDA	—	—	Rat cerebral cortex	CGS 19755	425	[^{3}H]CGP 39653	5 nM	L-glutamate (100 μM)	60 min/4°C
H1 (central)	—	17	Guinea-pig cerebellum	Pyrilamine	2.2	[^{3}H]pyrilamine	0.5 nM	triprolidine (100 μM)	10 min/22°C
H2	—	115	Guinea-pig striatum	Cimetidine	358	[^{125}I]APT	0.1 nM	tiotidine (100 μM)	150 min/22°C
H3	22	99	Rat cerebral cortex	(R)alpha-Me-histamine	1.6	[^{3}H](R)α-Me-histamine	0.5 nM	(R)α-Me-histamine (5 μM)	60 min/22°C
I2 (central)	—	48	Rat cerebral cortex	Idazoxan	6.3	[^{3}H]idazoxan	2 nM	cirazoline (1 μM)	30 min/22°C
M (non-selective)	28	100	Rat cerebral cortex	Atropine	0.25	[^{3}H]QNB	50 pM	atropine (1 μM)	120 min/22°C
N (central)	16	46	Rat cerebral cortex	Nicotine	30	[^{3}H]cytisine	1.5 nM	nicotine (10 μM)	75 min/4°C
5-HT1A (human)	—	71	human recombinant (CHO cells)	8-OH-DPAT	0.97	[^{3}H]8-OH-DPAT	0.3 nM	8-OH-DPAT (10 μM)	60 min/22°C
5-HT1D	—	69	Bovine caudate	Serotonin	2.3	[^{3}H]serotonin	2 nM	serotonin (10 μM)	30 min/22°C
5-HT2A (human)	—	27	Human recombinant (CHO cells)	Ketanserin	3.3	[^{3}H]ketanserin	2 nM	ketanserin (1 μM)	15 min/37°C
5-HT2C (humant)	—	85	Human recombinant (CHO cells)	Mesulergine	5.5	[^{3}H]mesulergine	0.7 nM	mesulergine (1 μM)	30 min/37°C
5-HT3 (human)	—	27	Human recombinant (HEK 293 cells)	MDL 72222	20	[^{3}H]BRL 43694	0.5 nM	MDL 72222 (10 μM)	60 min/22°C
5-HT4	12	109	Guinea-pig striatum	Serotonin	294	[^{3}H]GR 113808	0.1 nM	serotonin (30 μM)	30 min/22°C
5-HT6 (human)	—	—	Human recombinant (HEK 293 cells)	Serotonin	589	[^{3}H]LSD	2 nM	serotonin (100 μM)	60 min/37°C
5-HT7 (human)	10	83	Human recombinant (HEK 293 cells)	Serotonin	1.8	[^{3}H]LSD	4 nM	serotonin (10 μM)	120 min/22°C
5-HT uptake	—	84	Rat cerebral cortex	Zimelidine	113	[^{3}H]paroxetine	50 pM	serotonin (100 μM)	60 min/22°C

Abbreviations: CHO, chinese hamster overy cells; GABA, γ-aminobutyric acid; LSD, lysergic acid diethyl-amide.
—, no effects were seen at the indicated concentration.

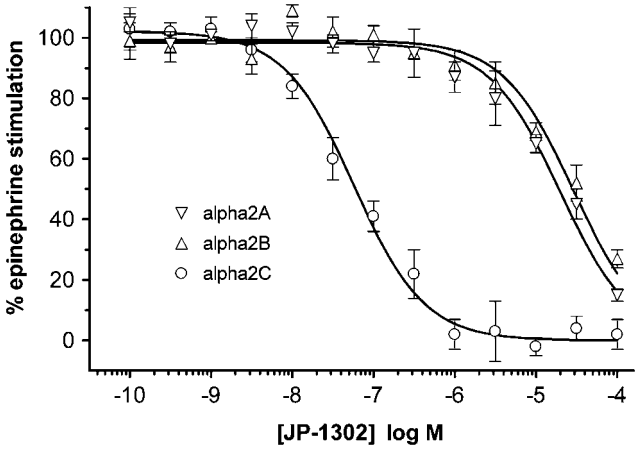

Figure 2 Subtype-selective antagonism of JP-1302 on human α_2-AR subtypes. [^{35}S]-GTPγS-binding was determined in membranes of CHO cells that were stably transfected with one of the three human α_2-subtypes (Pohjanoksa *et al*., 1997). The α_2-ARs in the membranes were stimulated with fixed concentrations of adrenaline that were chosen to represent the 6–7-fold of previously established EC_{50} values: 5 μM for the α_{2A} (EC_{50} = 0,76 μM), 15 μM for the α_{2B} (EC_{50} = 2.4 μM) and 5 μM for the α_{2C} (EC_{50} = 0.71 μM). In each experiment, every antagonist concentration was run in duplicate and each antagonism experiment was repeated at least three times.

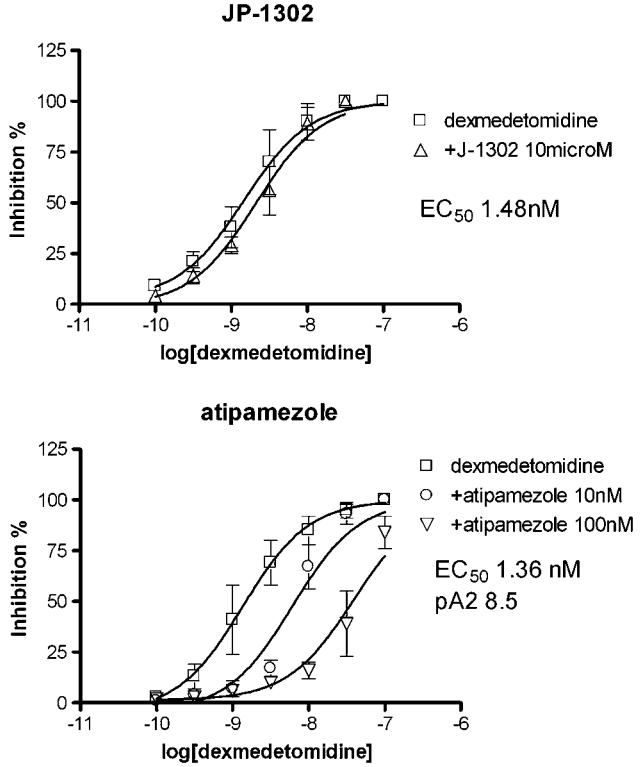

Figure 3 α_2-AR antagonism by atipamezole, but not JP-1302, on α_2-agonist (dexmedetomidine)-induced inhibition of contractions of vas deferens. The inhibition of electrically evoked contractions by dexmedetomidine was considered to be an indication of agonism at α_2-ARs and agonist effects were expressed as percent inhibition of the electrically evoked contractions. No agonism by JP-1302 was observed. Means and s.d. of replicates (n = 3 for atipamezole and n = 6 for JP-1302) were calculated and used to construct agonist dose–response curves in the absence and presence of antagonists. pA_2-values for antagonists were calculated as described in the Methods.

(Figure 5). A significant antagonistic effect for atipamezole was observed after 0.3 μmol kg^{-1} (P = 0.004, compared to vehicle + dexmedetomidine group) and higher doses. The complete reversal of hypothermia, that is, no statistical difference with the vehicle + vehicle-treated control group (P = 0.14), was observed only after the highest dose of atipamezole (3000 nmol kg^{-}1 s.c.).

Effect of JP-1302 in the FST for antidepressant activity. The effects on active behaviour of rats in the FST are shown in the Figure 6 as percentage of vehicle controls. One-way ANOVA revealed significant overall dose effects on the immobility time (P = 0.012) (Figure 6), where JP-1302 produced a dose-dependent decrease of immobility (P = 0.042 for the dose 1 μmol kg^{-1} and P = 0.004 for the doses 3 and 10 μmol kg^{-1}). The reference antidepressant drug desipramine decreased immobility after both studied doses (P = 0.012 and P < 0.001 for the doses 10 and 30 μmol kg^{-1}, respectively).

Effect of JP-1302 and atipamezole on acoustic startle and its prepulse inhibition. Atipamezole increased the magnitude of the startle response of PCP–treated SD rats by 92 ± 12% (mean ± s.e.m.) (P = 0.005; Mann–Whitney U-test) when the animals had not been exposed to a prepulse (Figure 7a), whereas the vehicle + vehicle or the JP-1302 + PCP-treated groups did not differ significantly from the vehicle-PCP group.

The data of PPI effects after pulses of 15 dB above background are presented in Figure 7b–d. PPI changes after the weaker prepulse levels (3 and 6 dB above background) were comparable to the results obtained with 15 dB prepulses; however, Kruskall–Wallis ANOVA did indicate statistically significant overall PPI effects (P < 0.05) only at the 15 dB prepulse level. Therefore, and for clarity, only these data are presented.

The PPI levels were reduced robustly by PCP treatment, as expected. The PCP-induced mean reduction of the calculated percentage PPI was 44.8 ± 10% (mean ± s.e.m.) (Figure 7b). Pretreatment with JP-1302 had a statistically significant reversing effect on PCP-disrupted PPI in two separate experiments conducted with SD rats (Figure 7b) and Wistar rats (Figure 7d). In the first experiment, the PPI% was 37.4% higher in the JP-1302 at 5 mg kg^{-1} + PCP group than that of the vehicle + PCP group (P < 0.001) (Figure 7b). In the second experiment, a dose-dependent and statistically significant (P < 0.05) PPI-enhancing effect was also seen in Wistar rats (Figure 7d), even though the effect was not as prominent and the mean potentiation of PPI after JP-1302 0.3, 1 and 3 μmol kg^{-1} was 15.4, 19.0 and 21.1%, respectively (P < 0.05). Atipamezole did not have effects on the calculated PPI% (P = 0.54) (Figure 7b). As in SD rats, JP-1302 also did not affect the magnitude of the startle effect in Wistar rats when the animals were not exposed to a prepulse (Figure 7c).

Discussion

The results presented in this paper show that JP-1302 is a selective α_{2C}-AR antagonist. The observed α_{2C}-AR binding

Table 3 Effects of JP-1302 (with no PCP treatments) on rat startle reactivity without prepulses, prepulse inhibition % and spontaneous locomotor activity of mice (cumulative counts of infrared beam breaks during 20 min)

Treatment	*Startle magnitude (pulse alone)*	*PPI% (at 15 dB PP intensity)*	*Locomotor activity (no. of beam brakes)*
Vehicle	46.5±6.7	64.6±4.4	91.3±11.8
JP-1302 3 μmol/kg	55.2±6.4	65.3±4.1	87.3±7.1
JP-1302 10 μmol/kg	55.4±7.5	70.2±4.3	85.6±4.8
JP-1302 30 μmol/kg	57.3±13	76.0±3.0*	88.5±5.8
JP-1302 100 μmol/kg	n.t.	n.t.	60.4±14.1
JP-1302 300 μmol/kg	n.t.	n.t.	5.9±2.5***

Abbreviations: JP-1302, acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine; n.t., not tested; PCP, phencyclidine.
The results are expressed as group mean±s.e.m. ($n=10$/group).
*$P<0.05$ and ***$P<0.001$ vs vehicle.

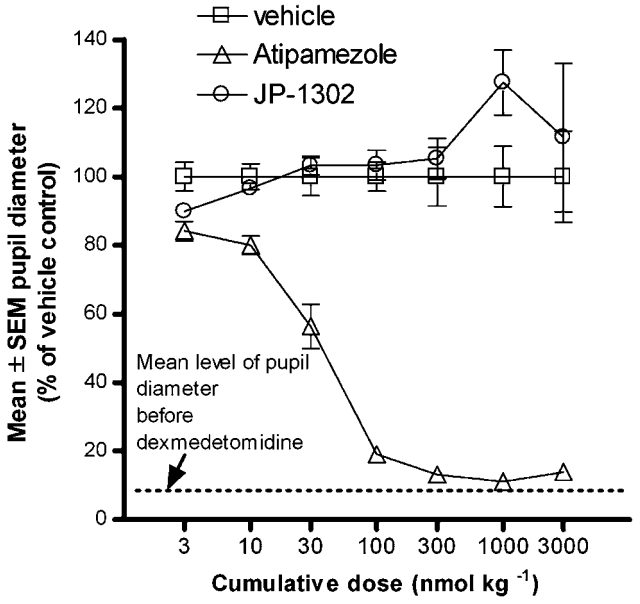

Figure 4 Antagonism of mydriatic effect of α_2-agonist as percentage of vehicle control. Dexmedetomidine produced a clear mydriatic effect (i.e. pupil dilatation) that was readily antagonized by atipamezole ($P<0.001$) but not by JP-1302. After measurement of the baseline pupil diameter, all rats ($n=5$/group, total of 15) were given the α_2-agonist dexmedetomidine 10 μg i.v. The mydriatic effect of dexmedetomidine was measured after 5 min and then cumulative doses of either atipamezole or JP-1302 or equivalent volumes of vehicle were applied intravenously at 5 min intervals.

and antagonism selectivity ratios of approximately 100-fold versus the α_{2A}- and α_{2B}-AR in cells expressing individual α_2-AR subtypes can be regarded remarkably high compared to the limited selectivity of other commonly used α_2-AR ligands in experimental research (Marjamäki *et al*., 1993; Jansson *et al*., 1994; Jasper *et al*., 1998; Mayer and Imbert, 2001). For example, when using essentially the same methods as in this paper, the α_{2C}-preferring antagonist MK-912 displayed only fivefold selectivity for α_{2C} vs α_{2A} receptor (Marjamäki *et al*., 1993).

Although some other ligands have been reported to possess somewhat higher selectivity ratios among the α_2-AR subtypes, their use has been limited by additional and often predominating pharmacological activities on other targets. Such nonspecific compounds include chlorpromazine, yohimbine and prazosin with reported α_{2C}/α_{2A} ratios of 9, 30 and 28, respectively (Marjamäki *et al*., 1993). Although these compounds can be utilized in some special experimental settings, their use for *in vivo* evaluations are compromised by their multiplicity of pharmacodynamic actions such as dopamine D2 antagonism (chlorpromazine and yohimbine) or potent α_1-antagonism (prazosin and yohimbine) (Mayer and Imbert, 2001). In addition, for many compounds the α_{2C} vs α_{2B} selectivity ratios are typically even lower than their α_{2C}/α_{2A} ratios.

From a practical experimental point of view, the differentiation between the α_{2B} and the α_{2C} subtypes may not appear as important as that of the α_{2C} vs the α_{2A}, as the α_{2A}-AR is known to be the major subtype in the regulation of physiological and pharmacological functions. Nevertheless, when trying to study the functional role of the α_{2C}-AR in separation from that of the other α_2-AR subtypes, it is highly desirable that actions on the α_{2B} subtype can also be avoided. A profiling campaign for JP-1302 among 30 known drug targets, in particular other G protein-coupled receptors, did not identify any additional binding sites with affinities comparable to that on the α_{2C}-AR. Although this outcome does not definitively rule out the possibility that some as yet unknown secondary site may have contributed to the *in vivo* actions of JP-1302, it does strongly support the notion that the observed effects of JP-1302 are grounded in its α_{2C}-AR antagonism.

JP-1302 reduced the immobility in the FST, that is, it displayed clear antidepressant-like efficacy. This finding is well in line with studies employing transgenic mouse lines, as α_{2C}-KO mutant mice were more active and α_{2C}-OE mice were less active in the FST. Interestingly, α_{2A}-KO mice have been reported to display increased immobility in the FST and the loss of the α_{2A}-AR has been suggested to elicit a depressant response (Schramm *et al*., 2001). Indeed, in normal rats subtype non-selective α_2-antagonists when given alone were not effective in the FST and the anti-immobility actions of desipramine were blocked by co-administration of an α_2-antagonist (Cervo *et al*., 1990; Kauppila *et al*., 1991). Taken together, these findings suggest that it is not only the α_{2C}-antagonistic action, but also the absence or minimization of an α_{2A}-AR blocking effect, that is, allowing for a stimulating input via the α_{2A}-AR, that seems to be important for achieving the antidepressant-like effects by α_2-antagonist in the FST.

JP-1302 also reversed the PCP–induced PPI defect, that is, it had antipsychotic-like effect. This effect could not have clearly been predicted from the results of transgenic mouse studies where α_{2C}-KO and α_{2C}-OE mutations were associated

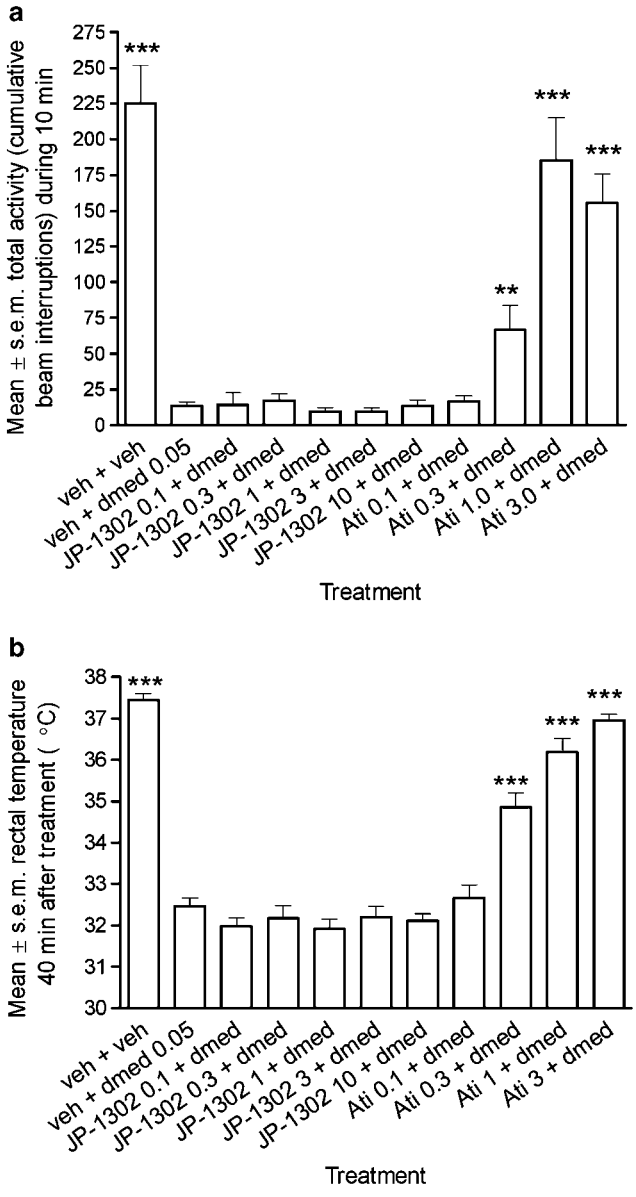

Figure 5 Antagonism of α_2-agonist-induced sedation (**a**) and hypothermia (**b**). Atipamezole dose-dependently reversed the immobility and hypothermia produced by dexmedetomidine whereas JP-1302 had no effect. The animals were injected either with JP-1302 or atipamezole 20 min before the injection of dexmedetomidine (50 nmol kg^{-1} s.c.). Spontaneous locomotor activity was measured 20–30 min after dexmedetomidine injection and the body temperature was recorded at the end of the locomotor test. $^{**}P<0.01$, $^{***}P<0.001$ compared to vehicle + dexmedetomidine group. Data are presented as mean ± s.e.m., $n=8$–12/group.

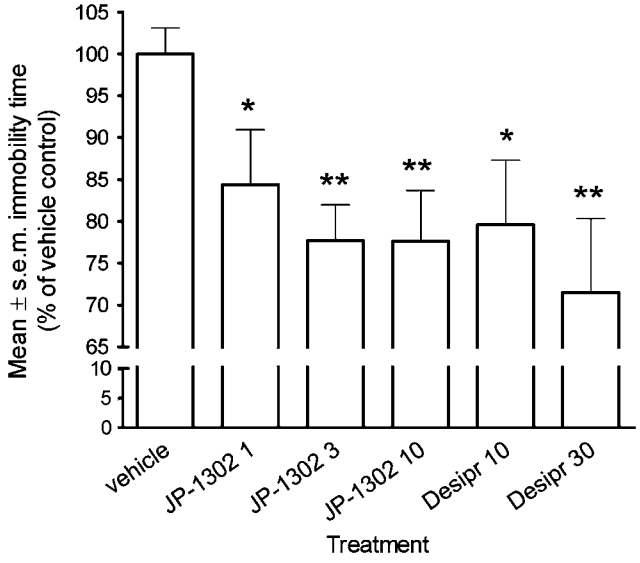

Figure 6 Effect of JP-1302 and reference antidepressant desipramine (Desipr) in the forced swimming test. Drug treatments were applied during the period between the two sessions, the first 15 min after the pre-test swim and the second dosing at 1 h before the test swim. The effects on immobility are presented as mean ± s.e.mean, $n=10$–16/group. $^{*}P<0.05$, $^{**}P<0.01$, $^{**}P<0.001$ compared to vehicle group.

with decreased and increased PPI, respectively, both in drug-naïve and in PCP–treated mice (Sallinen *et al*., 1998). Actually, the straightforward prediction from these observations would have been to expect α_{2C}-agonists, not antagonists, to display antipsychotic-like efficacy in the PPI model. It appears that in the case of the PPI paradigm, this kind of direct extrapolation would have been confounded by some physiological compensatory changes in the complex regulatory network of mice carrying the lifelong α_{2C}-AR mutation, whereas such compensatory changes appear not as critical for the functions related to the FST. Possibly owing to their rather subtle nature in the present context, such compensatory changes appear to be very difficult to predict. Nevertheless, our current results further support a significant role of α_{2C}-ARs in the modulation of sensorimotor gating mechanisms. Interestingly, JP-1302 did not have a significant effect on the magnitude of the startle response, whereas atipamezole, which did not have an effect on PPI, greatly enhanced startle reactivity. In this respect, it seems that the subtype selectivity of α_2-antagonists again plays a critical role in the PPI model.

As already stated by Kalkman and Loetscher (2003), the antipsychotic compounds clozapine, risperidone, quetiapine and iloperidone display higher affinity for the α_{2C}-AR compared to the α_{2A}-AR (Schotte *et al*., 1996; Kalkman and Loetscher, 2003), that is, their α_2-AR balance is shifted towards the α_{2C} subtype. These compounds, however, also have significant interactions with several other receptors, such as dopamine D2 and 5-hydroxytryptamine receptors that are the main targets of the majority of current antipsychotics. Therefore, it has not been clear to what extent the α_2-AR antagonism of these compounds, particularly in form of their preference for the α_{2C}-AR, may play a role in their antipsychotic efficacy and other clinical profile. The current study supports the idea that α_{2C}-antagonism might participate in the therapeutic effect of these compounds, as our results strongly suggest that α_{2C}-antagonism plays a significant role in the PPI phenomenon, which is considered to be predictive for the therapeutic efficacy of antipsychotics (Swerdlow *et al*., 1994). This finding still needs confirmatory studies, for example in form of clinical

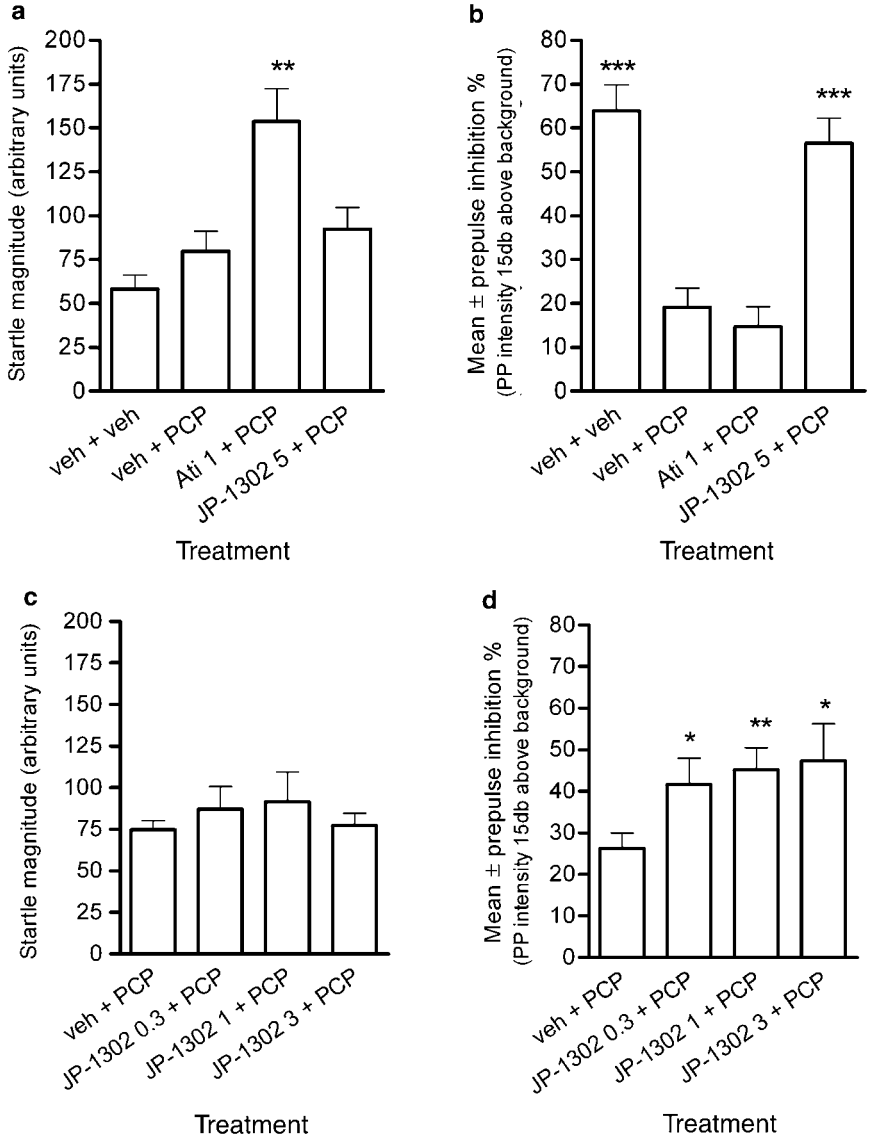

Figure 7 Effect of JP-1302 and atipamezole (Ati) on startle reactivity (**a**, **c**) and PPI as percentage inhibition of the startle reflex to 118dB pulse alone (**b**, **d**) in two separate experiments with SD (**a**, **b**) and Wistar (**c**, **d**) rats. Pulse and prepulse durations were 40 ms, the prepulse level was 15 dB above background noise and the prepulse–pulse interval was 100 ms. Data are expressed as mean ± s.e.m., $n = 8$–10. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle + phencyclidine (PCP) group.

investigations with an α_{2C}-AR antagonist in psychiatric disorders. Other studies, such as exploring the effects of α_{2C}-AR antagonists on cognitive processes and dopamine D2-antagonist-induced extrapyramidal side effects, are also warranted, as these effects have been proposed to possibly be related to the α_{2C}-AR antagonistic activity of the above-mentioned compounds (Kalkman *et al*., 1998; Arnsten, 2004).

JP-1302 was not able to antagonize the α_2-agonist-induced sedation, mydriasis or hypothermia, and JP-1302 did not have any detectable effect on the α_2-agonist-induced inhibition of vas deferens contractions. All these effects were effectively antagonized by the subtype nonselective α_2-antagonist atipamezole (Haapalinna *et al*., 1997). The antagonism by atipamezole but not JP-1302 in these models was expected, as these actions have previously been suggested to be mediated by α_{2A}-ARs and not α_{2C}-ARs. The presynaptic α_{2C}-AR has been associated with a different mode of stimulus control compared to the presynaptic α_{2A}-AR (Hein *et al*., 1999). Further, there is evidence for some functional role of α_{2C}-AR in the modulation of hypothermia and vas deferens contractions in α_{2C}-KO mice (Sallinen *et al*., 1997; Altman *et al*., 1999) and therefore the observed absence of any apparent antagonistic responses in these assays after JP-1302 treatment may be considered somewhat unexpected.

The neuronal circuits and mechanisms that might be participating in the described effects of JP-1302 in the FST and PPI assays cannot be estimated from the current data. However, it can be speculated that both the striatum and the hippocampus are likely to be involved: both of these areas are known to be critical in the modulation of sensorimotor

gating (PPI) and in the development of behavioural despair and the expression of α_{2C}-ARs is concentrated in these areas. Based on the observation of differences in dopamine and 5-hydroxytryptamine levels in the brains of mice with genetically altered expression of α_{2C}-ARs (Sallinen *et al*., 1997), it is possible that JP-1302 may have modulatory effects on the balance of these monoamines through α_{2C}-heteroreceptors located in non-noradrenergic neurons (Holmberg *et al*., 1999). It is equally well possible, that a blockade of the autoreceptor function of α_{2C}-AR in noradrenergic neurons may increase noradrenaline release, which in turn may stimulate other types of adrenoceptors, including an increase in the activity of α_{2A}-autoreceptors (Trendelenburg *et al*., 2001). These kinds of complex interactions in different brain areas could explain the observed outcomes in the FST and PPI and the significance of α_2-subtype-selectivity.

In conclusion, the current study shows that JP-1302 is a valuable tool for studies exploring the role of the α_{2C}-AR. It is hypothesized that the α_{2C}-AR has significant potential as a target for novel treatments of neuropsychiatric disorders (MacDonald *et al*., 1997; Scheinin *et al*., 2001; Kalkman and Loetscher, 2003; Arnsten, 2004) and highlights the importance of the subtype selectivity of α_2-antagonists. New studies with JP-1302 as well as with other specific compounds highly selective for α_{2C}-ARs are needed to clarify the role of the α_{2C}-AR in the CNS further as well in the periphery, such as the cardiovascular system, where it also appears to play some, though as yet not completely clear, modulator role (Brede *et al*., 2004; Regitz-Zagrosek *et al*., 2006).

Conflict of interest

The authors state no conflict of interest.

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