ACS Chemical Neuroscience

Research Article

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JNJ-40255293, a Novel Adenosine A_{2A}/A_1 Antagonist with Efficacy in Preclinical Models of Parkinson's Disease

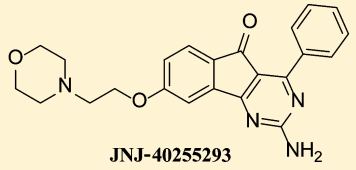
John R. Atack,[‡] Brian C. Shook,*[,†] Stefanie Rassnick,[†] Paul F. Jackson,[†] Kenneth Rhodes,[†] Wilhelmus H. Drinkenburg,[‡] Abdallah Ahnaou,[‡] Paula te Riele,[‡] Xavier Langlois,[‡] Brian Hrupka,[‡] Patrick De Haes,[‡] Herman Hendrickx,[‡] Nancy Aerts,[‡] Koen Hens,[‡] Annemie Wellens,[‡] Jef Vermeire,[‡] and Anton A. H. P. Megens[‡]

[†]Janssen Research and Development, Welsh and McKean Roads, Spring House, Pennsylvania 19477, United States

[‡]Janssen Research and Development, Turnhoutseweg 30, Beerse B-2340, Belgium

Supporting Information

ABSTRACT: Adenosine A_{2A} antagonists are believed to have therapeutic potential in the treatment of Parkinson's disease (PD). We have characterized the dual adenosine A_{2A}/A_1 receptor antagonist JNJ-40255293 (2-amino-8-[2-(4-morpholinyl)ethoxy]-4-phenyl-5*H*-indeno-[1,2-*d*]pyrimidin-5-one). JNJ-40255293 was a high-affinity (7.5 nM) antagonist at the human A_{2A} receptor with 7-fold in vitro selectivity versus the human A_1 receptor. A similar A_{2A}:A_1 selectivity was seen in vivo (ED_{50}'s of 0.21 and 2.1 mg/kg p.o. for occupancy of rat brain A_{2A} and A_1 receptors, respectively). The plasma EC_{50} for occupancy of rat brain A_{2A} receptors was 13 ng/mL. In sleep–wake encephalographic (EEG) studies, JNJ-40255293 dose-dependently enhanced a consolidated waking associated with a subsequent delayed compensatory sleep (minimum effective dose: 0.63 mg/kg p.o.). As measured by microdialysis, JNJ-40255293 did not affect dopamine and noradrenaline release in the prefrontal cortex and the striatum. However, it was able to reverse effects (catalepsy, hypolocomotion, and conditioned avoidance impairment in rats; hypolocomotion in mice) produced by the dopamine D_2 antagonist haloperidol. The compound also potentiated the agitation induced by the dopamine agonist apomorphine. JNJ-40255293 also reversed hypolocomotion produced by the dopamine-depleting agent reserpine and potentiated the effects of L-dihydroxyphenylalanine (L-DOPA) in rats with unilateral 6-hydroxydopamine-induced lesions of the nigro-striatal pathway, an animal model of Parkinson's disease. Extrapolating from the rat receptor occupancy dose–response curve, the occupancy required to produce these various effects in rats was generally in the range of 60–90%. The findings support the continued research and development of A_{2A} antagonists as potential treatments for PD.



JNJ-40255293

Human adenosine receptor functional activity (EC_{50}, nM)

	A_1	A_{2A}	A_{2B}	A_3
JNJ-40255293	48 ± 16	6.5 ± 3.8	230 ± 92	9200

KEYWORDS: *Parkinson's disease, adenosine, A_{2A} antagonists, neuropharmacology, dopamine, neurodegeneration*

Parkinson's disease (PD), a neurodegenerative disorder affecting 1–2% of people over the age of 65,[1] is characterized by motor dysfunction often accompanied by mood disorders and cognitive dysfunction.[2] The motor impairment is primarily associated with a loss of dopaminergic nigrostriatal neurons. Several approaches have been designed to compensate for the loss of dopamine, including dopamine replacement therapy with the dopamine precursor L-dihydroxyphenylalanine (L-DOPA) to increase the production of dopamine. Although L-DOPA remains the gold standard, additional dopamine-related therapies include dopamine receptor agonists (e.g., apomorphine) and inhibition of dopamine metabolism via inhibition of monoamine oxidase type B or catechol *O*-methyl transferase.[3] Although very useful for treating the motor dysfunction associated with PD, however, dopamine-related therapies can produce side effects, such as dyskinesias and on–off effects, which become progressively more severe as treatment continues.[4] Nondopaminergic therapies,[5] particularly those relating to glutamate and adenosine,[6] represent a potential alternative treatment approach that may overcome these side effects.

Adenosine acts via a family of four different G-protein-coupled receptor subtypes, namely, A_1, A_{2A}, A_{2B}, and A_3.[7] The A_1 and A_3 subtypes couple to the G*i* family of G proteins whereas the A_{2A} and A_{2B} receptors couple via the G*s* family.[7a] The adenosine receptors provide therapeutic potential for either agonists or antagonists, both in the periphery and the CNS.[8] The well-known stimulant effects of caffeine are, at least partially, related to its weak nonselective antagonist actions at adenosine receptors.[7b,9] It is still not clear to what extent the pharmacological

Received: July 27, 2014
Revised: September 8, 2014
Published: September 9, 2014

properties of caffeine can be attributed to the individual adenosine receptor subtypes (A_1, A_{2A}, and A_{2B}) with which it interacts.[9]

Regarding PD, the A_{2A} receptor subtype has received particular attention,[10] based upon its preferential localization within the striatum,[11] more specifically in the striatal enkephalin-containing GABAergic neurons that project to the globus pallidus and comprise the so-called indirect pathway.[12] Within these neurons, A_{2A} receptors are coexpressed with dopamine D_2 receptors where the two receptor types may exist as heterodimers.[13] The role of the adenosine A_{2A} receptor in PD-related motor dysfunction is further underscored by the fact that binding of the A_{2A}-selective positron emission tomography ligands [^{11}C]SCH442416 and [^{11}C]TMSX is altered in PD-associated dyskinesia, whether the dyskinesia was L-DOPA-induced or not.[14]

Compared with the A_{2A} subtype, adenosine A_1 receptors have a more widespread distribution within the CNS, being highly expressed in the cortex, hippocampus, cerebellum, thalamus, and striatum.[14b] Acting at presynaptic autoreceptors, A_1 receptors inhibit the release of several neurotransmitters.[7b,15] Within the striatum, presynaptic A_1 receptors are expressed on the terminals of glutamatergic and dopaminergic afferents, controlling the release of glutamate and dopamine, respectively.[16] The adenosine A_1 receptor has been implicated in cognition with, for example, A_1 antagonists reversing cognitive deficits induced either by scopolamine[17] or by lesions of the nucleus basalis magnocellularis.[15]

In the present study, we describe the in vitro and in vivo characteristics of JNJ-40255293 (Figure 1), an arylindenopyrimidine that is a novel, high affinity antagonist at adenosine A_{2A} receptors and a moderate-high affinity antagonist of the A_1 subtype.[18] Comparisons were made with istradefylline (KW-6002),[19] ASP5854,[20] and JNJ-27631734 (compound **1**).[21] These three compounds are all high-affinity A_{2A} antagonists, with istradefylline being a low-affinity antagonist and the other two being high-affinity A_1 antagonists. In addition, ASP5854 was able to reverse scopolamine induced memory deficits in rodents, while the selective istradefylline was not. The doses used in this study were selected based on previously published data and by screening for the active dose level in the current assays.

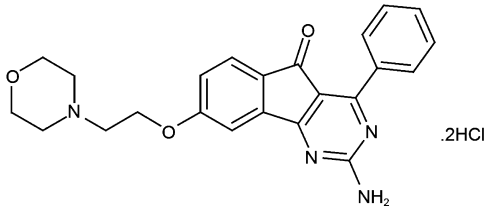


Figure 1. Structure of 2-amino-8-[2-(4-morpholinyl)ethoxy]-4-phenyl-5*H*-indeno[1,2-*d*] pyrimidin-5-one (JNJ-40255293).

RESULTS

Binding Affinity and Functional Activity of JNJ-40255293 at Human Recombinant Adenosine Receptors. The 7.5 nM binding affinity of JNJ-40255293 for the A_{2A} subtype of human recombinant adenosine receptors was comparable to the functional activity of 6.5 nM (Table 1). The binding affinity of JNJ-40255293 at the other adenosine receptor subtypes was not measured. As the functional activities of JNJ-40255293, istradefylline, and ASP5854 were generally in good agreement with the available binding affinities; however, the ratio A_{2A} to A_1 functional activities of JNJ-40255293 (6.5 and 48 nM, respectively) suggests that this compound is a modestly (7-fold) selective A_{2A} versus A_1 antagonist.

Table 1. Binding Affinities and Functional Activities of JNJ-40255293, Istradefylline, and ASP5854 to Human Adenosine A_1, A_{2A}, A_{2B} and A_3 Receptors[a]

human recombinant adenosine receptor binding affinity (K_i, nM)[b]				
	A_1	A_{2A}	A_{2B}	A_3
JNJ-40255293	N/A	7.5 ± 4.9	N/A	430 ± 150
istradefylline	656 ± 150	26 ± 5	829 ± 120	658 ± 380
ASP5854	11 ± 7	3.1 ± 1.5	N/A	>10 000
human recombinant adenosine receptor functional activity (EC_{50}, nM)				
	A_1	A_{2A}	A_{2B}	A_3
JNJ-40255293	48 ± 16	6.5 ± 3.8	230 ± 92	9200
istradefylline	181 ± 90	6.8 ± 2.0	440 ± 220	688
ASP5854	3.8 ± 2.6	4.9 ± 1.9	250 ± 140	>10 000

[a]Values shown are either single values or the mean + SD of 3−5 separate experiments. [b]N/A = Not assayed.

In functional assays, neither JNJ-40255293, istradefylline, nor ASP5854 demonstrated any agonist efficacy on either the human recombinant A_1, A_{2A}, A_{2B}, or A_3 receptor. On the other hand, all three compounds antagonized the effects of adenosine at the A_{2A} subtype, with respective EC_{50} values for JNJ-40255293, istradefylline and ASP5854 being 6.5, 6.8, and 4.9 nM. Regarding the A_1 subtype, JNJ-40255293 had a functional activity at this subtype of 48 nM, which was around 7-fold lower than at the A_{2A} subtype. In comparison, istradefylline had a much lower EC_{50} for the A_1 compared to A_{2A} subtype (respective EC_{50} values =181 and 6.8 nM). In contrast to JNJ-40255293 and istradefylline, ASP5854 had equivalent EC_{50} values at the A_1 and A_{2A} subtypes (3.8 and 4.9 nM, respectively). Finally, in a panel of over 50 different receptor, enzyme and transporter binding assays, JNJ-40255293 did not have appreciable affinity (i.e., IC_{50} values >1 μM) for any binding site other than adenosine receptors (see Supporting Information Table 1).

Rat Brain Adenosine Receptor Occupancy. JNJ-40255293 produced dose-dependent occupancy of rat brain A_{2A} receptors (Figure 2A) with the ED_{50} being calculated as 0.21 mg/kg. The occupancy of adenosine A_1 receptors was lower than that of A_{2A} receptors with the ED_{50} for A_1, 2.1 mg/kg, being 10-fold higher than that for A_{2A} receptors. Hence, the 10-fold difference in in vivo A_{2A} versus A_1 ED_{50} (Table 2) reflects the difference in the in vitro A_{2A} versus A_1 selectivity (Table 1). Occupancy of rat brain adenosine A_{2A} receptors by JNJ-40255293 was also time-dependent (Figure 2B) with maximum occupancy being observed 1 h after p.o. dosing and, at this time point, doses of 0.63 and 2.5 mg/kg produced occupancy of 84 and 94%, respectively.

Compared with istradefylline, JNJ-40255293 possessed a similar level of rat brain adenosine A_{2A} receptor occupancy (Table 2), with an ED_{50} of 0.19 mg/kg p.o. for istradefylline and an ED_{50} of 0.21 mg/kg p.o. for JNJ-40255293. Istradefylline demonstrated negligible occupancy of rat brain A_1 receptors (<15%) after a dose of 10 mg/kg p.o. (which corresponded to an ~95% occupancy of A_{2A} receptors). ASP5854 had an in vivo A_{2A} versus A_1 selectivity of around 10-fold (respective ED_{50} values of 0.073 and 0.75 mg/kg p.o.) and in this regard was similar to JNJ-40255293 albeit the potency of ASP5854 was approximately 3-fold greater than that of JNJ-40255293 (nonoverlapping 95% confidence intervals; Table 2).

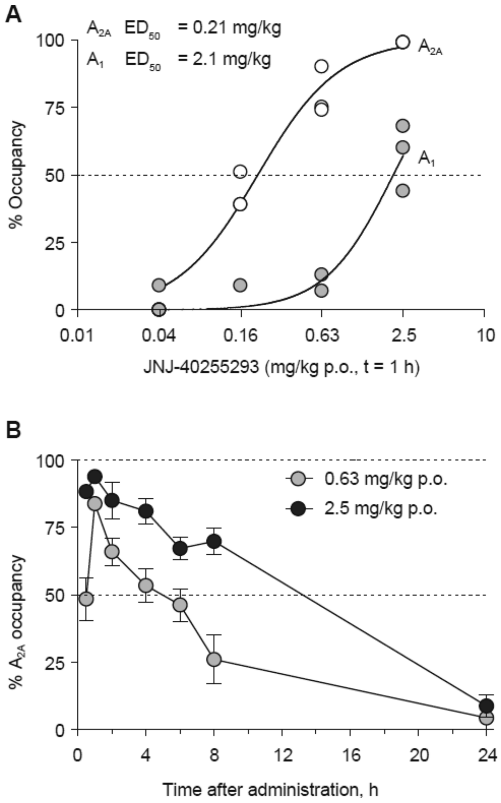


Figure 2. Rat brain adenosine receptor occupancy by JNJ-40255293. (A) Adenosine A_1 and A_{2A} receptor occupancy was measured 1 h after p.o. dosing with JNJ-40255293 (0.04−2.5 mg/kg) using [^{3}H]DPCPX and [^{3}H]SCH-58261 to label A_1 and A_{2A} receptors in vivo, respectively. Data points represent values for individual animals. (B) Time course of adenosine A_{2A} occupancy was measured 0.5−24 h after p.o. dosing with 0.63 or 2.5 mg/kg of JNJ-40255293. Data shown are the mean ± SEM (n = 3−5/group).

Plasma Concentration−Occupancy Relationships in Rat. Terminal blood samples were collected from satellite rats dosed as part of the occupancy time-course studies described above (Figure 2B). Plasma samples were prepared and drug concentrations measured by LC-MS/MS. Occupancy values were plotted as a function of plasma drug concentrations of JNJ-40255293 (Figure 3). From these data, the plasma drug concentration required to occupy 50% of rat brain A_{2A} receptors was estimated to be 13 ng/mL for JNJ-40255293 (Table 2). Similar data were also derived for istradefylline and ASP5854 which had respective plasma EC_{50} values for A_{2A} occupancy of 10 and 20 ng/mL (Table 2). The corresponding EC_{50} value for drug concentrations in the brain were 19, 23, and 6.7 ng/g for JNJ-40255293, istradefylline, and ASP5854, respectively, indicating that the brain/plasma ratio of JNJ-40255293 is in the region of 1.5 whereas those for istradefylline and ASP5854 were 2.3 and 0.33, respectively.

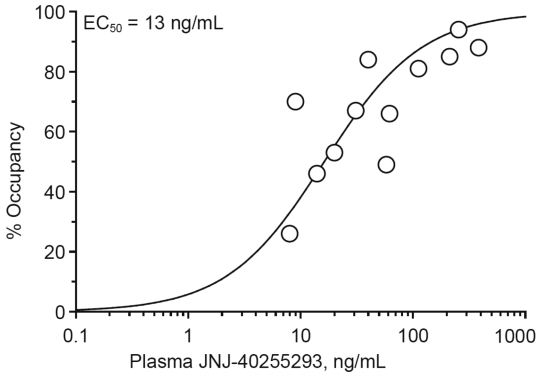


Figure 3. Relationship between rat brain A_{2A} receptor occupancy and plasma drug concentrations of JNJ-40255293. Analyses were performed in satellite groups of animals dosed as part of the occupancy time-course experiments. Each data point represents a single animal.

JNJ-40255293 Had No Effect on Dopamine Release. JNJ-40255293 at the dose of 2.5 mg/kg, which produces >90% occupancy of A_{2A} receptors and around 50% occupancy of A_1 receptors (Figure 2), did not affect extracellular dopamine and noradrenaline concentrations in either the prefrontal cortex or the striatum (Figure 4). This is in contrast to D-amphetamine (2.5 mg/kg) and methylphenidate (20 mg/kg), both of which produced robust outflow of dopamine and noradrenaline in both the prefrontal cortex and the striatum. The effects of JNJ-40255293 on dopaminergic hypofunctioning are thus not accompanied by the massive release of dopamine seen with dopaminergic behavioral stimulants, which may result in decreased abuse liability.

JNJ-40255293 Elicited Active Waking in Rats. JNJ-40255293, at doses of 0.04 and 0.16 mg/kg, did not elicit significant changes in vigilance states during the first 4 h of the recording session (Figure 5A). However, the mixed model ANOVA revealed that JNJ-40255293 from the doses of 0.63 mg/kg onward dose-dependently enhanced active waking (treatment × time interaction, $F(9, 212) = 7.77$, $p < 0.0001$) at the expense of light sleep (treatment × time interaction, $F(9, 212) = 3.00$, $p < 0.005$), deep sleep (treatment × time interaction, $F(9, 212) = 5.39$, $p < 0.0001$), and REM sleep (treatment × time interaction, $F(9, 212) = 4.39$, $p < 0.0001$) (Figure 5B, only active wake and REM sleep displayed). JNJ-40255293 produced more consolidated wakefulness, as indicated by longer average duration of continuous bouts of waking, while importantly no homeostatic rebound of different sleep stages was observed during the dark phase of the circadian time. In addition, the wake-promoting effects of JNJ-40255293 are consistent with dose-dependent increases in sleep onset latencies. Notably, no indications of sleep fragmentation were revealed by examination of total number of transitions from sleep states toward waking (data not shown).

JNJ-40255293 Reverses Haloperidol-Induced Catalepsy. In mice, JNJ-40255293 induced a highly significant ($p <$

Table 2. Summary of Data Derived from Rat Brain Adenosine A_1 and A_{2A} Receptor Occupancy Studies

	ED_{50} (95% confidence limits) (mg/kg, p.o., t = 1 h)		A_{2A} occupancy EC_{50} values	
	A_1	A_{2A}	plasma (ng/mL)	brain (ng/g)
JNJ-40255293	2.1 (1.7−2.7)	0.21 (0.17−0.27)	13	19
Istradefylline	>10[a]	0.19 (0.16−0.25)	10	23
ASP5854	0.75 (0.50−1.1)	0.073 (0.059−0.090)	20	6.7

[a]15% occupancy at 10 mg/kg.

dx.doi.org/10.1021/cn5001606 | ACS Chem. Neurosci. 2014, 5, 1005−1019

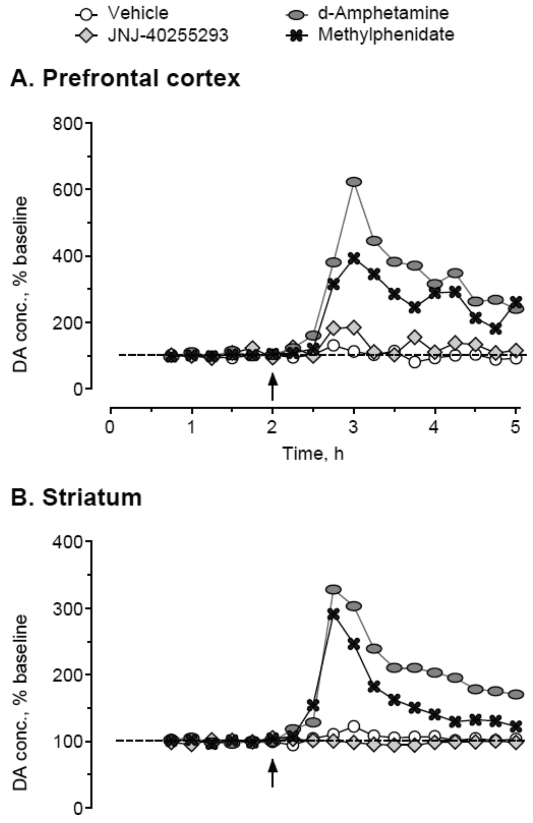


Figure 4. In vivo microdialysis in the nucleus accumbens (upper panel) and prefrontal cortex (bottom panel). Sprague–Dawley rats were orally administered vehicle (n = 14) and a single dose of JNJ-40255293 (2.5 mg/kg, n = 12), D-amphetamine (2.5 mg/kg, n = 12), and methylphenidate (20 mg/kg, n = 13). Dopamine levels were determined from brain dialysates collected in 30 min samples from prefrontal cortex (upper panel) and striatum (bottom panel) and expressed relative to baseline. Data are expressed as median relative to changes of mean individual baseline.

0.001) dose-dependent reversal of haloperidol-induced catalepsy when administered 0.5 h after the haloperidol injection (Figure 6, left panel). The minimal effective dose was 0.1 mg/kg, and the graded ED_{50} was <0.1 mg/kg. A dose of 10 mg/kg significantly reversed haloperidol-induced catalepsy for at least 4 h postadministration (Figure 6, right panel).

In male Wistar rats, JNJ-40255293 dose-dependently reversed haloperidol (0.63 mg/kg, s.c.) induced catalepsy that was scored (0–6) 2, 3, and 4 h after the haloperidol injection in rats cotreated with test compound immediately prior to the haloperidol injection (Figure 7, left panel). Comparative data obtained with istradefylline and ASP-5854 are also shown (Figure 7, middle and right panel, respectively). The dotted horizontal line in each graph represents the critical level for reversal of catalepsy. Corresponding ED_{50}'s and 95% confidence limits are given in the inset in each graph. Table 3 compares the ED_{50}'s of the compounds at time of peak effect.

Comparison of Effects of JNJ-40255293, Istradefylline, and ASP5854 in Dopaminergic Challenge Models. Table 3 compares the ED_{50}'s of JNJ-40255293, istradefylline, and ASP5854 obtained for reversal of catalepsy in rats (at time of peak effect; see Figure 7) and in a number of additional dopamine challenge models (for dose–response relations, see Supporting Information Figures 1–4), together with corresponding A_{2A} receptor occupancy levels (estimated by interpolating or extrapolating from the A_{2A} occupancy dose–response curves (see Figure 2A for JNJ-40255393; Table 2). The estimated occupancy values show that despite the differing A_{2A} to A_1 selectivity of JNJ-40255293 and ASP5854 relative to istradefylline there was no consistent trend with JNJ-40255293 and ASP5854 to require markedly different levels of occupancy compared to istradefylline, with all three compounds having ED_{50}'s in the different assays that corresponded to between ~30–90% A_{2A} occupancy. Hence, it would appear that the A_1 component of JNJ-40255293 and ASP5854 does not contribute appreciably to efficacy in these dopaminergic challenge models.

Figure 8 plots the ED_{50} of JNJ-40255293 for prolongation of apomorphine (1.0 mg/kg i.v.)-induced agitation as a function of time after s.c. and p.o. dosing. JNJ-40255293 dose- and time-dependently potentiated the apomorphine-induced agitation (ED_{50}'s at peak: 0.54 mg/kg, p.o.; 0.21 mg/kg, s.c.) and showed a fast onset (<0.5 h, s.c. and p.o.) and a relatively short duration of action (2.7 h, p.o.; 2.5 h, s.c.).

JNJ-40255293 Reverses Reserpine-Induced Akinesia. Figure 9 shows that, in both male CF-1 mice and male Wistar rats, reserpine (0.6 mg/kg, s.c.) produces a marked reduction in locomotor activity. In both species, this akinesia was reversed in a dose-dependent manner by JNJ-40255293. More specifically, in mice, a dose of 0.10 mg/kg gave a nonsignificant partial reversal, whereas at 1.0 mg/kg the reversal was essentially complete. Similarly, in rats, a dose of 0.10 mg/kg was also ineffective, whereas doses from 1.0 mg/kg and above significantly reversed the reserpine-induced deficits; at 10 mg/kg, the effect was comparable to that obtained with the positive control JNJ-27631734 (10 mg/kg, p.o.).

JNJ-40255293 Potentiates the Effects of L-DOPA in 6-OHDA-Lesioned Rats. In this model, asymmetry in motor behavior is produced in animals with unilateral dopamine depletion in the nigrostriatal pathway in the CNS.[22] Figure 10A shows that, during the 1 h pretreatment interval prior to L-DOPA administration, JNJ-40255293 was able to produce a modest, dose-dependent increase in the number of contralateral rotations observed in 6-OHDA-lesioned rats, implying that JNJ-40355293 alone was able to provide a modest enhancement of dopamine neurotransmission. In contrast, L-DOPA (10 mg/kg p.o.) was able to produce a more pronounced enhancement in the number of contralateral rotations (Figure 10B). JNJ-40255293 was able to dose-dependently enhance the effects of L-DOPA with the minimum efficacious dose (MED) for this effect being 1 mg/kg p.o., which corresponds to respective adenosine A_1 and A_{2A} receptor occupancies of 22% and 91%.

JNJ-40255293 Is Not Proconvulsant in Rats. In the 1 h period prior to pentylenetetrazole administration, JNJ-40255293 induced mild behavioral stimulant effects (sniffing, rearing, excitation), but there were no signs of overt seizure activity. Following the administration of pentylenetetrazole, there were no significant differences between JNJ-40255293 and vehicle in corresponding ED_{50}s of pentylenetetrazole for several scoring levels (>0, >1, >2, or >3) of tremors or clonic or tonic seizures (overlapping 95% confidence intervals) (Figure 11).

Drug Discrimination Studies. Drugs that increase dopamine concentrations in the brain, such as amphetamine and cocaine, have rewarding properties (i.e., they make the animal "feel good"), an effect mediated via the dopamine reward pathway. Furthermore, nondopaminergic drugs that possess rewarding properties, such as LSD, chlordiazepoxide, and fentanyl, also increase dopaminergic neurotransmission in the dopamine reward pathway. Since amphetamine and JNJ-

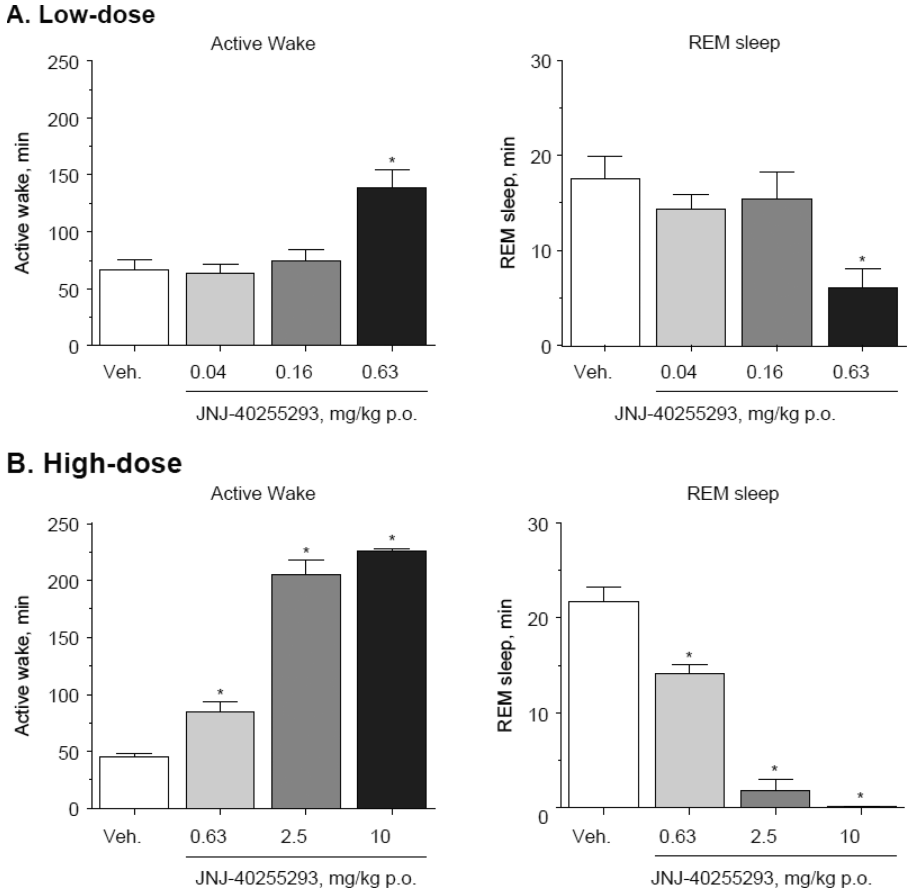


Figure 5. Effects of JNJ-40255293 on sleep−wake EEG in rats. Time spent in the states of active waking and REM sleep during the first 4 h of the registration session following oral administration of (A) vehicle ($n = 8$) and low (0.04−0.63 mg/kg, $n = 8$ for each treatment condition) or (B) vehicle ($n = 8$) and high (0.63−10 mg/kg, for each treatment condition). Values are presented as means ± SEM. *Significantly different from vehicle, mixed ANOVA model.

40255293 both enhance dopaminergic neurotransmission, albeit via different mechanisms (increased dopamine release and enhanced postsynaptic signaling via heterodimeric A_{2A}/D_2 receptors, respectively), JNJ-40255293 was evaluated in a drug discrimination assay to assess whether the interoceptive cues (i.e., the "feeling") produced by JNJ-40255293 resembled those produced by amphetamine. Additional studies were performed to assess whether JNJ-40255293 generalized to other drugs known to have rewarding properties. Finally, studies were carried out to assess whether preadministration of JNJ-40255293 could block the rewarding properties of the drugs tested.

In these assays, JNJ-40255293 neither generalized to nor antagonized any of the various drugs tested, emphasizing that it has a mechanism distinct not only from amphetamine but also from a wide variety of other drugs with rewarding properties (Table 4).

■ DISCUSSION

As degeneration of nigro-striatal dopaminergic neurons is the underlying cause of PD,[23] dopamine augmentation has become the first line therapy in PD.[24] Although the dopamine precursor L-DOPA or direct dopamine agonists (e.g., bromocriptine, pergolide, pramipexole, ropinirole, piribedil, cabergoline, apomorphine, lisuride) have undoubted therapeutic efficacy, they also have side effects such as somnolence, hallucinations, insomnia, nausea, and constipation.[29] Accordingly, potential alternative approaches, which indirectly modulate dopaminergic function, have emerged, such as blockade of adenosine A_{2A} receptors, which functionally oppose dopamine D_2 receptors.[25]

Initial efforts to obtain an A_{2A} antagonist were based on the purine adenosine, for example, ST-1535,[26] on the xanthines caffeine and theophylline, for example, istradefylline (KW-6002),[27] and on nonxanthines, for example, SCH-58261.[28] Subsequent work identified the nonxanthine pyrazolotriazolo-pyrimidines, for example, SCH-442416 and SCH-420814 (preladenant),[29] the bicyclic triazolotriazine ZM241385 (also showing appreciable A_{2B} affinity,[30] and the triazolopyrimidine BIIB014 (V2006, vipadenant).[31]

In the present Article, we describe the properties of the arylindenopyrimidine JNJ-40255293 (compound **14**).[18] The respective functional affinities of JNJ-40255293 for human adenosine A_1 and A_{2A} receptors were 48 and 6.5 nM, reflecting modest A_{2A} versus A_1 selectivity. This selectivity is similar to that of the purine analogue ST-1535 that progressed into clinical studies.[31,32] The human A_{2A} and A_1 affinities of 11 and 5.6 nM for ASP5854 observed in the present study are comparable to previously published values of 9.0 and 1.8 nM, with these latter affinities being comparable at the human, mouse, and rat receptors.[20] Species differences have been reported for the affinity of certain ligands, with, for example, 8-phenyl-substituted xanthines having lower affinity for rat than for human receptors.[33] Although we did not directly compare the affinity of JNJ-40255293 against human and rat receptors, the in vivo occupancy data suggests that the modest A_{2A} versus A_1 binding

dx.doi.org/10.1021/cn5001606 | ACS Chem. Neurosci. 2014, 5, 1005−1019

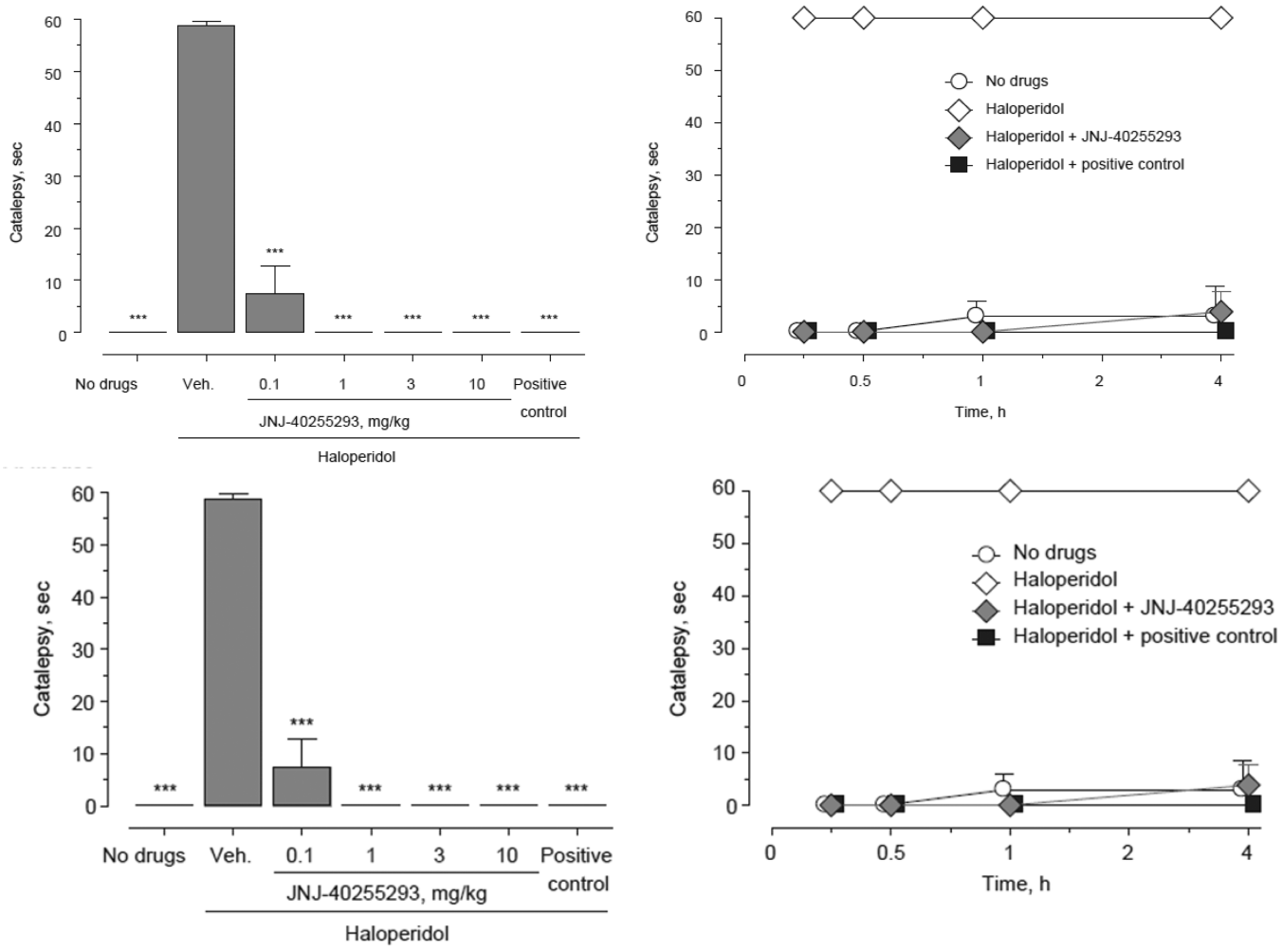


Figure 6. JNJ-40255293 reverses haloperidol-induced catalepsy in mice. Male Balb/c mice were pretreated with haloperidol (1.0 mg/kg s.c.) and 0.5 h later dosed with either vehicle, JNJ-40255293 (0.1, 1, 3, and 10 mg/kg p.o.) or positive control JNJ-27631734 (10 mg/kg, p.o.). After a further 0.5 h, the time spent in a cataleptic position was measured over a 60-s test period and values shown are mean + SEM (n = 8/group). The right-hand panel shows that, in a separate experiment, the effects of JNJ-40255293 (10 mg/kg, p.o.) were evident whether catalepsy was measured 0.5, 1, 2, or 4 h after drug treatment. Asterisks indicate significant differences as compared with the haloperidol + vehicle control treatment group (***P < 0.001, Dunnett's test of multiple comparisons).

selectivity in human recombinant receptors is retained in native rat brain receptors.

The fact that three compounds (JNJ-40255293, istradefylline, and ASP5854) with A_{2A} antagonism but different degrees of A_1 antagonist activity all produced similar effects, both qualitatively and quantitatively, against a variety of behavioral phenomena corresponding to different states of central dopaminergic neurotransmission (blockade of D_2 receptors by haloperidol, stimulation of D_1 and D_2 receptors with apomorphine, depletion of dopamine using reserpine, or lesions of the nigro-striatal dopaminergic neurons with 6-hydroxydopamine) suggests that all of these dopamine modulatory effects are mediated via the A_{2A} subtype. This result is in line with previous data showing that both the A_{2A}-selective antagonist istradefylline and the non-selective adenosine antagonist caffeine reversed effects of haloperidol whereas the A_1 antagonist DPCPX was without effect.[34]

PD is characterized by significant motor dysfunction and various nonmotor disturbances such as cognitive deficits. These cognitive deficits are, at least partially related, to the hypo-functioning of the central dopaminergic system. The haloperidol-induced impairment of the conditioned avoidance response might be an interesting model for studying both motor and cognitive deficits associated with hypo-functioning of the dopaminergic system. The ability of adenosine A_2 antagonists to alleviate both the motor deficits and the cognitive deficits related to hypo-functioning of the dopaminergic system in this model suggests therapeutic potential against both the motor and the cognitive deficits in PD.

In the drug discrimination assay, JNJ-40255293 did not generalize to other dopamine-enhancing drugs such as amphetamine or cocaine, in contrast to previous data which showed that the adenosine A_{2A} antagonist MSX-3 (as well as the A_1 antagonist CPA) generalized to cocaine and methamphetamine.[35] Although it is possible that the rewarding properties of drugs of abuse may be modulated by adenosine A_{2A} antagonists,[36] JNJ-40255293 did not antagonize the effects of several other drugs of abuse in the present study. This lack of antagonism of the interoceptive cues associated with various drugs of abuse suggests that JNJ-40255293 would have little therapeutic benefit in drug addiction.[37] At the very least, the data showing that JNJ-40255293 does not generalize to a wide-range of dopaminergic and nondopaminergic drugs in the drug discrimination assay is consistent with JNJ-40255293 having a mechanism distinct from

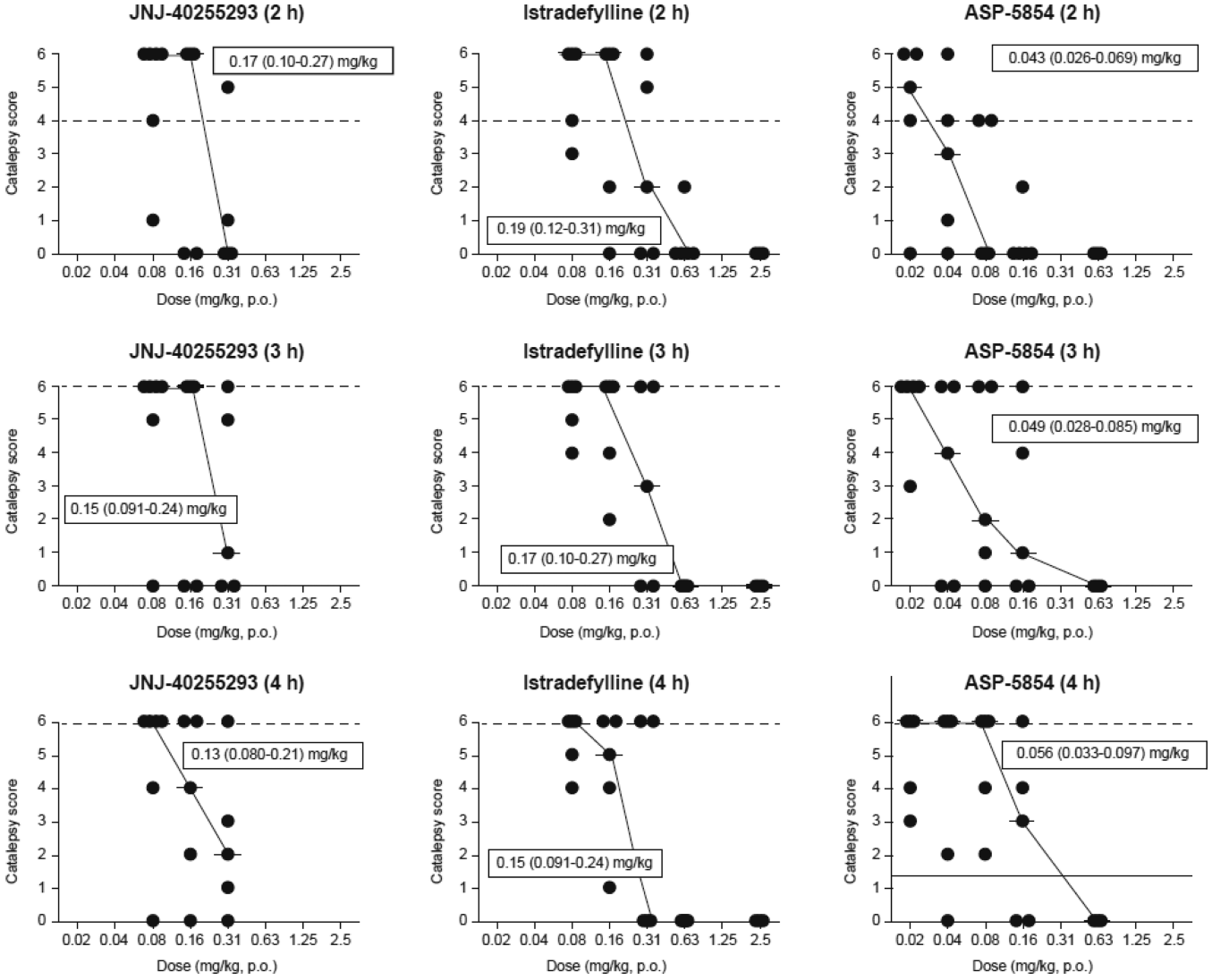


Figure 7. Reversal of haloperidol (0.63 mg/kg s.c.)-induced catalepsy in Wistar rats after oral cotreatment with JNJ-40255293, istradefylline, and ASP-5854. Catalepsy was scored (0−6) at 2, 3, and 4 h after haloperidol injection. Test compound or vehicle was administered immediately prior to the haloperidol injection. Shown are individual and median values (symbols and stripes, respectively) per dose group (n = 5). The dotted horizontal lines represent the critical levels adopted for reversal of catalepsy that were used for the determination of the ED_{50}'s shown in the insets and also listed in Table 3.

these various compounds and reduces the risk of abuse liability. A low abuse liability is further supported by the observation that JNJ-40255293 did not produce conditioned-place preference (data not shown), indicating it does not have pronounced rewarding properties. Nevertheless, these collective data do not rule out the possibility that JNJ-40255293 may have rewarding properties that are best assessed using the self-administration paradigm.

Given the role of A_1 receptors in inhibiting dopamine release,[7b,16b] we hypothesized that the antagonist activity of JNJ-40255293 at A_1 receptors might result in an increase in dopamine that would complement the enhanced postsynaptic D_2 receptor function produced by A_{2A} antagonism. However, we have shown that JNJ-40255293 was not more potent than the pure A_{2A} antagonist in a variety of dopaminergic-related pharmacological models. In addition, our in vivo microdialysis results demonstrated that JNJ-40255293 at dose of 2.5 mg/kg p.o., which corresponds to ∼50% A1 occupancy, had no effect on extracellular concentration of dopamine in striatum and prefrontal cortex. The lack of effect on dopamine outflow in the striatum might suggests a low potential for abuse liability.

Adenosine acts as an endogenous somnogen that regulates sleep drive.[38] Genetic knockouts of A_1 and A_{2A} and pharmacological studies have demonstrated that A_1 receptors are clearly not required for the promotion of wakefulness.[39] Thus, it is unlikely that A_1 receptors play a major role in the arousal properties of JNJ-40255293, at least at the minimum effective dose (0.63 mg/kg) at which A_1 receptor occupancy is <20%. Therefore, the changes observed in sleep−wake behavior are most likely mediated by the A_{2A} receptor since similar patterns of changes (increased active awake and decreased sleep states) were observed with the selective A_{2A} antagonist istradefylline.

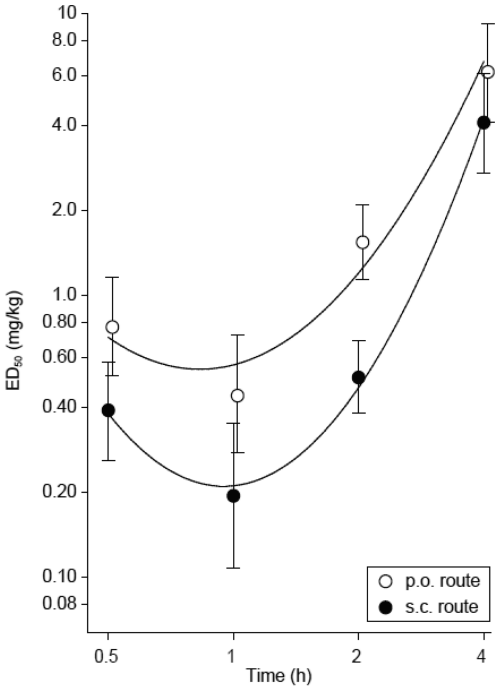
A_1-selective antagonists have been reported to enhance cognitive performance in rodents.[17a,40] Accordingly, A_1 antagonism is thought to be responsible for the beneficial effects of the mixed A_{2A}/A_1 antagonist ASP5854 in reversing a scopolamine-induced deficit in rat passive avoidance, whereas the A_{2A} selective

dx.doi.org/10.1021/cn5001606 | ACS Chem. Neurosci. 2014, 5, 1005−1019

Table 3. ED_{50}'s (and 95% confidence intervals; mg/kg, p.o.) of JNJ-40255293, Istradefylline, and ASP5854 in Various Dopaminergic Challenge Models Together with Estimated A2A Occupancy Levels

		ED_{50} (95% CL; mg/kg p.o.) [A_{2A} occupancy, %][a]		
assay	species	JNJ-40255293	istradefylline	ASP5854
haloperidol-induced catalepsy, reversal[b]	rat	0.13 [33%] (0.080−0.21)	0.15 [43%] (0.091−0.24)	0.043 [32%] (0.026−0.069)
haloperidol-induced hypolocomotion, reversal[c]	rat	1.2 [93%] (0.72−1.89)	0.51 [79%] (0.30−0.88)	0.19 [76%] (0.12−0.31)
haloperidol-induced hypolocomotion, reversal[d]	mouse	0.67 [NC] (0.50−0.91)	0.89 [NC] (0.72−1.1)	0.056 [NC] (0.046−0.069)
haloperidol-induced conditioned avoidance impairment, reversal[e]	rat	0.44 [74%] (0.23−0.85)	0.89 [89%] (0.72−1.1)	0.13 [68%] (0.087−0.19)
apomorphine-induced agitation, potentiation[f]	rat	0.44 [74%] (0.28−0.72)	1.5 [94%] (1.03−2.3)	0.29 [84%] (0.18−0.48)

[a]Dose−response relations are available in the Supporting Information Figures 1−4. NC Not calculated (mouse brain occupancy was not measured and therefore estimates of mouse brain occupancy could not be made). [b]Haloperidol (0.63 mg/kg s.c.)-induced catalepsy in rats was scored 2−4 h after injection; test compound was administered p.o. immediately prior to the haloperidol injection. ED_{50} at time of peak effect is listed. [c]Haloperidol (0.16 mg/kg i.v.)-induced hypolocomotion in rats was measured for the first 0.5 h immediately after injection; test compound was administered p.o. One h before haloperidol injection. [d]Haloperidol (0.31 mg/kg i.v.)-induced hypolocomotion in mice was measured for the first 0.5 h immediately after injection; test compound was administered p.o. 0.5 h before haloperidol injection. [e]Haloperidol (0.63 mg/kg, s.c.)-induced conditioned avoidance impairment in rats was measured at 60, 90, and 120 min after injection; test compound was administered p.o. immediately before haloperidol injection. The median response latency over the three time intervals was used for further evaluation. [f]Apomorphine (1.0 mg/kg i.v.)-induced agitation (licking, sniffing, chewing) in rats was scored every 5 min for the first hour after injection with test drug being administered p.o. One h before apomorphine injection.



	P.O. route	S.C. route
Time of peak effect (h):	0.83	0.95
Peak-effect dose (mg/kg):	0.54	0.21
Onset of action (h)[a]:	< 0.5	< 0.5
Duration of action (h)[a]:	2.7	2.5

[a]Determined at 4 times the peak-effect dose

Figure 8. ED_{50} (and 95% confidence limits) of JNJ-40255293 for prolongation of apomorphine (1.0 mg/kg i.v.)-induced agitation as a function of time after p.o. and s.c. dosing. Curves of best fit through the data points were plotted by nonlinear regression of the polynomial second order function $y = a + bx + cx^2$.

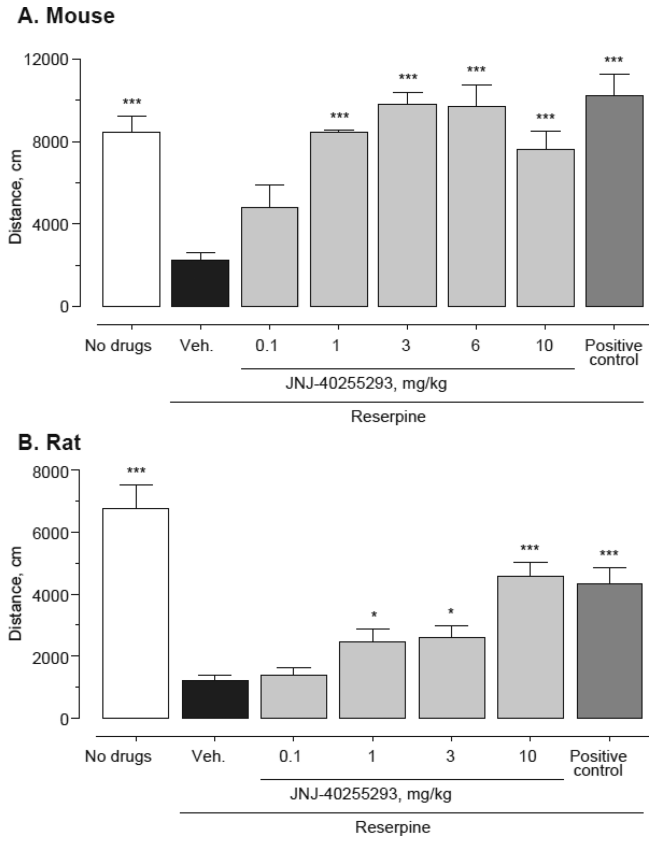


Figure 9. JNJ-40255293 reverses reserpine-induced akinesia in both A. Mice and B. Rats. At 18 h following pretreatment with reserpine, animals were dosed p.o. with either vehicle, JNJ-40255293 or, as a positive control, JNJ-27631734 (10 mg/kg). Either 0.5 h (mice) or 1 h (rats) later, locomotor activity was measured over a 0.5 h period. Values shown represent mean + SEM of total locomotor activity measured during the 0.5 h period (n = 14−16 and n = 16−18 for mouse and rat dose−response studies, respectively). Asterisks indicate significant differences as compared with the reserpine + vehicle control group (*P < 0.05 and ***P < 0.001, Hochberg test of multiple comparisons).

antagonist istradefylline was not effective in the same paradigm.[20] Thus, it is possible that the A_1 antagonist component of JNJ-40255293 might have beneficial effects on cognitive function. Further experiments may reveal the potential contribution of the A_1 antagonist component of JNJ-40255293 on cognitive processes.

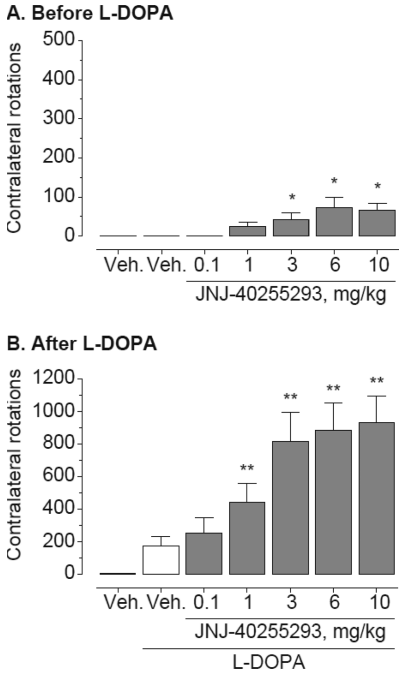


Figure 10. Data showing the effects of JNJ-40255293 on the number (mean ± SEM) of contralateral rotations in rats with unilateral 6-OHDA lesions of the nigrostriatal pathway. Data show the total number of contralateral rotations measured: (A) during the 1 h pretreatment interval between dosing of JNJ-40255293 and dosing of L-DOPA (10 mg/kg, p.o.) together with carbidopa (2.5 mg/kg, p.o.) and (B) during the first 2 h period after L-DOPA administration. The effects of L-DOPA are dose-dependently increased by oral administration of JNJ-40255293, with the MED of JNJ-40255293 being 1 mg/kg, p.o. Asterisks indicate significant differences as compared with the L-DOPA + vehicle control treatment group (*P < 0.05, Tukey's test of multiple comparisons). Values shown are mean ± SEM; n = 16/group.

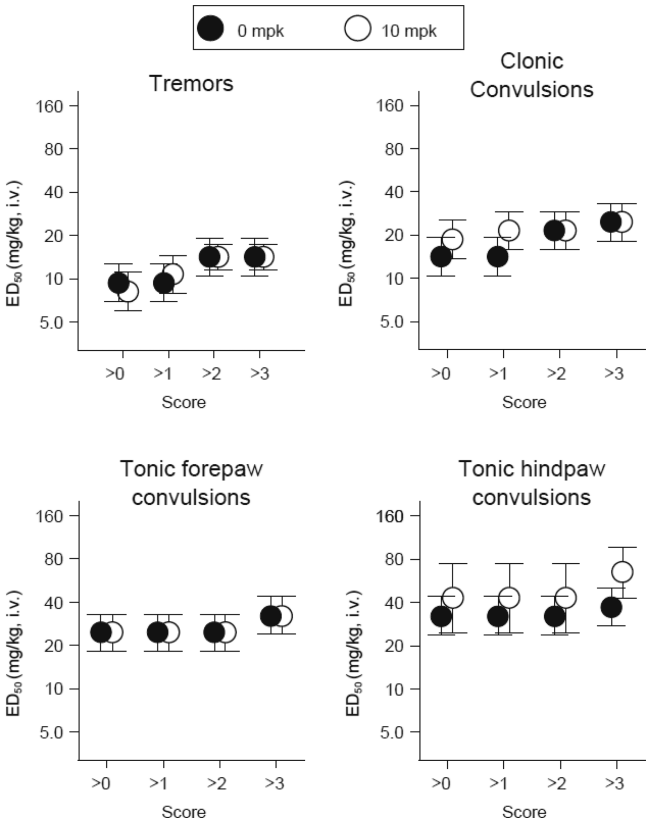


Figure 11. ED_{50} of pentylenetetrazole (PTZ) required to produce tremors and clonic or tonic convulsions was measured in rats pretreated for 1 h with either vehicle or JNJ-40255293 (10 mg/kg p.o.). Overlapping 95% confidence intervals between corresponding ED_{50}'s attest to absence of proconvulsant effect of JNJ-40255293.

Table 4. Lack of Effect of JNJ-40255293 in the Drug Discrimination Assay

drug[a]	mechanism	generalization[b]	antagonism[b]
amphetamine	dopamine-releasing agent	no effect	no effect
chlordiazepoxide	$GABA_A$ receptor modulator	no effect	no effect
cocaine	dopamine reuptake inhibitor	no effect[c]	no effect
DOM	serotonergic agonist	no effect	no effect
ethanol	general CNS depressant	no effect	no effect
fentanyl	opioid agonist	no effect[c]	no effect
LSD	serotonergic agonist	no effect	no effect
nicotine	nicotinic acetylcholine receptor agonist	no effect	no effect

[a]DOM = 1-(2,5-Dimethoxy-4-methylphenyl)-2-aminopropane. LSD = Lysergic acid diethylamide. [b]Data refer to doses of up to 10 mg/kg s.c., a dose at which dose occupancy of A_{2A} receptors would be essentially 100%. [c]At higher doses (80 mg/kg s.c.), there was a weak, partial generalization to cocaine and fentanyl, but the pharmacological relevance of this effect, if any, is unclear.

JNJ-40255293 demonstrated good pharmacokinetic properties in Balb/c mice, Sprague–Dawley rats, and Cynomolus monkeys with respective half-lives after i.v. dosing of 0.8, 0.8, and 4.9 h that were comparable to the half-lives after oral dosing of 1.1, 1.2, and 5.5 h.[18] The compound also had moderate-to-good oral bioavailability (61, 18 and 55% in mice, rat and monkey, respectively). Based on the robust preclinical pharmacology and good pharmacokinetic properties, JNJ-40255293 was progressed into preclinical development. However, development was halted due to toxicity that was distinct from the genotoxicity observed with the structurally related JNJ-27631734.[18,21b] More specifically, in a 1 month rat study, there was decreased body weight and a generally poor condition resulting in death in some animals. These changes were accompanied neuropathologically by localized neuronal necrosis and edema in the hippocampal formation and basal piriform cortex. In addition, a phospholipidosis-like pathology was observed in a dog 1 month study. It is unclear to what extent the preclinical toxicity, especially the neuronal necrosis, was mechanism-related.

Among the various A_{2A} antagonists undergoing clinical trials, the xanthine istradefylline was the most advanced and appreciable levels of A_{2A} occupancy were demonstrated in man.[41] Although istradefylline demonstrated signs of efficacy in reducing OFF time in PD patients with motor fluctuations, the US FDA, in the absence of two positive phase III studies, issued a "not approvable" letter in 2008.[42] Further studies with compounds such as SYN115[43] should help clarify the role of A_{2A} antagonists in the treatment of PD whereas the clinical evaluation of the JNJ-40255293-like modest 12-fold A_{2A} versus A_1 selectivity profile of ST-1535[26a] should help establish the beneficial roles, if any, of blockade of the A_1 receptor over and above antagonism of the adenosine A_{2A} receptor alone.[32]

dx.doi.org/10.1021/cn5001606 | ACS Chem. Neurosci. 2014, 5, 1005–1019

Overall, JNJ-40255293 demonstrated remarkable potential in experimental models of PD. The findings support the promising anti-PD effects obtained by blocking A_{2A} receptors.

METHODS

Drugs. JNJ-40255293 (2-amino-8-[2-(4-morpholinyl)ethoxy]-4-phenyl-5*H*-indeno[1,2-*d*]pyrimidin-5-one; Figure 1; compound **14**[18]) and 2-amino-4-phenyl-8-(pyrrolidin-1-ylmethyl)-5*H*-indeno[1,2-*d*]-pyrimidin-5-one (JNJ-27631734, compound **1**[18]) were synthesized as described elsewhere.[21a] 8-((*E*)-2-(3,4-Dimethoxyphenyl)ethenyl)-1,3-diethyl-7-methyl-3,7-dihydro-1*H*-purine-2,6-dione (istradefylline; KW-6002), 5-[5-amino-3-(4-fluorophenyl) pyrazin-2-yl]-1-isopropylpyridine-2(1H)-one (ASP5854), DOM, haloperidol, and fentanyl were obtained from internal synthesis. All other compounds were obtained from external sources: 6-hydroxydopamine, apomorphine, carbidopa, L-DOPA, nicotine, reserpine, and pentylenetetrazole (Sigma-Aldrich, St. Louis, MO or Diegem, Belgium), chlordiazepoxide (Sequoia Research Products; U.K.), cocaine (Belgopia, Brussels, Belgium), D-amphetamine (Certa SA, Eigenbrakel, Belgium), ethanol (Merck, Overijse, Belgium), LSD (Lipomed, Arlesheim, Switzerland), and methylphenidate (Sigma-Aldrich, Saint Louis, MO). [^{3}H]DPCPX and [^{3}H]CGS21680 were purchased from PerkinElmer, Inc. [^{3}H]ZM-241385 was obtained from Tocris Cookson, U.K. [^{125}I]AB-MECA and [^{3}H]SCH-58261) were purchased from GE Healthcare Europe GMBH (Diegem, Belgium).

JNJ-40255293 was dissolved in 20% hydroxypropyl-β-cyclodextrin for the reserpine and 6-hydroxydopamine tests in rats, in 0.5% Methocel for the reserpine test in mice, and in 10% hydroxypropyl-β-cyclodextrin for all other tests. 6-Hydroxydopamine was dissolved in saline containing 0.1% ascorbic acid. Apomorphine, chlordiazepoxide, cocaine, D-amphetamine, ethanol, DOM, LSD, nicotine, and pentylenetetrazole were dissolved in distilled water. JNJ-27631734 was dissolved in 0.5% Methocel. Carbidopa and L-DOPA were suspended in 0.5% Methocel, methylphenidate was prepared in acidified distilled water, reserpine in 0.5% acetic acid, ASP5854 in acidified 20% hydroxypropyl-β-cyclodextrin, haloperidol in acidified saline or acidified distilled water, and [^{3}H]DPCPX and [^{3}H]SCH-58261 in saline. Istradefylline was suspended in distilled water containing 1% polysorbate 80. The formulations were stored at room temperature in closed containers protected from light. They were subcutaneously (s.c.), orally (p.o.), or intraperitoneally (i.p.) administered in volumes of 10 mL/kg. Intravenous (i.v.) injections of challenge agents were done in a volume of 2 mL/kg. Vehicles were also tested to control for solvent-related effects.

In Vitro Binding. Receptor binding studies were performed using [^{3}H]DPCPX, [^{3}H]CGS21680, [^{3}H]ZM-241385, and [^{125}I]AB-MECA as radioligands to selectively bind to human recombinant A_1, A_{2A}, A_{2B}, and A_3 receptors stably expressed in either CHO or HEK cell lines. The concentration of JNJ-40255293, istradefylline, and ASP5854 required to inhibit radioligand binding by 50% was determined and from these IC_{50} values, the K_i values were calculated using the Cheng-Prusoff equation using GraphPad Prism (GraphPad Software, San Diego, CA).

In Vitro Functional Activity. Adenosine receptors modulate intracellular cAMP levels, with A_1 and A_3 receptors decreasing concentrations via a G_i coupled mechanism that inhibits adenylate cyclase and with A_{2A} and A_{2B} receptors increasing cAMP concentrations via a G_s-mediated stimulation of adenylate cyclase. Stably transfected cell lines were established (CHO for A_1, A_{2A}, and A_3 and HEK-293 for A_{2B} adenosine receptors) and the ability of JNJ-40255293, istradefylline, and ASP5854 to attenuate adenosine-induced changes in cAMP concentrations was measured using either a cAMP-response element (CRE)-linked β-galactosidase reporter assay or by radioimmunoassay of intracellular cAMP concentrations.

In Vivo Studies. All experimental protocols were carried out in strict accordance with the European Communities Council Directive of 24th November 1986 (86/609/EEC) and were approved by the ethical committee of Janssen Research and Development. Animals were housed in ventilated cages and were maintained under controlled environmental conditions: 22 °C ± 2 °C ambient temperature, relative humidity 60%, standard 12:12 light cycle regime, and free access to standard laboratory food chow and tap water.

Rat Brain Receptor Occupancy. Male Wistar rats (Crl:WI; Charles River, Sulzfeld, Germany; approximately 220 g) were dosed p.o. with either vehicle (water) or various doses (0.04, 0.16, 0.63, or 2.5 mg/kg) of JNJ-40255293, istradefylline, or ASP5854. Rats were sacrificed 1 h after dosing. At 10 or 15 min before sacrifice, the rats received tail vein injections of [3H]SCH-58261 or[3H]DPCPX (10 μCi/rat); radioligands that selectively label adenosine A_{2A} or A_1 receptors in vivo.[44] After sacrifice, brains were removed from the skull and rapidly frozen. Then 20 μm thick brain sections were cut using a cryostat-microtome, thaw-mounted on microscope slides, and loaded into a β-imager to quantify the amount of radioactivity in brain area expressing high levels of receptor, that is, the striatum for the A_{2A} receptor and the thalamus for the A_1 receptor. Nonspecific binding was defined by the extent of radioligand binding in brain area virtually devoid of one or the other receptor: the whole cerebellum for the A_{2A} receptor and the pons for the A_1 receptor. Occupancy was calculated as the extent by which specific binding in drug-treated animals was reduced relative to vehicle-treated rats. From the dose–response studies, the concentration required to achieve 50% occupancy (ED_{50}) was estimated (see Statistics subsection). Following the ED_{50} measurement, a time course of JNJ-40255293 A_{2A} receptor occupancy was performed with animals being sacrificed at specific time points (0.5, 1, 2, 4, 8, and 24 h) after dosing (0.63 and 2.5 mg/kg p.o.). The A_{2A} occupancy was determined as described above.

Satellite groups of animals (n = 3/time point) were dosed with JNJ-40255293 (0.63 or 2.5 mg/kg dissolved in water) or istradefylline (0.63 or 2.5 mg/kg suspended in Methocel/Tween), and animals were euthanized and blood was collected 0.5, 1, 2, 4, 6, 8, and 24 h postdose. Terminal blood samples and samples of brain were collected. Plasma was prepared, frozen, and stored at −80 °C, along with brain samples, for subsequent bioanalysis using LC-MS/MS.

In Vivo Microdialysis. Adult, male Sprague–Dawley rats (Harlan; The Netherlands) were singly housed, then surgically implanted with two guide cannulas (CMA Microdialysis AB, Kista, Sweden); one into the left nucleus accumbens (NAC) and the other into the right prefrontal cortex (PFC) using methods described in more detail elsewhere.[45] One day prior to testing, rats were transferred to the experimental room and inserted with microdialysis probes (PFC with 4 mm and NAC with 2 mm membrane length) containing a 20 kD cutoff polyarylethersulfone membrane. Starting at 7:00 AM on the next experimental day, dialysates were collected in four consecutive 30 min samples, followed by eight consecutive 30 min samples postadministration of vehicle or JNJ-40255293 (2.5 mg/kg p.o.), D-amphetamine (2.5 mg/kg p.o.), or methylphenidate (20 mg/kg p.o.). Norepinephrine (NE), dopamine (DA), and serotonin (5-HT) levels were determined from brain dialysates by high-performance liquid chromatography, and data were analyzed as the area under curve after vehicle or different treatments.

Sleep–Wake EEG Study in Rats. Sleep polysomnographic variables were determined during 20 h in 32 adult male Sprague–Dawley rats (Harlan; The Netherlands), which were chronically implanted with electrodes for recording the cortical electroencephalogram (EEG), electrical neck muscle activity (EMG), and ocular movements (EOG) using methods described in more detail elsewhere.[46] In two consecutive studies including 32 animals divided into 4 groups of 8 subjects, vehicle or JNJ-40255293 (0.04, 0.16, and 0.63 mg/kg, p.o., first study) and vehicle or JNJ-40255293 (0.63, 2.5, and 10 mg/kg, p.o., second study) were administered during the light phase of the 12 h light/12 h dark cycle, at 1:30 pm. In the case of animal reuse, a wash-out period of least 2 weeks was allowed to elapse between experiments to avoid long-lasting and rebound effects on sleep. The online acquisition was performed during 20 h using a bipolar recorder system (Embla, Medcare, Iceland), amplified, and digitized at a sample rate of 200 Hz. Treatment of the signals was managed by using a software package (Somnologica, MedCare, Iceland), which turned the computer into a polygraphic workstation for signal recording. The EEG signals were high-pass filtered at 0.5 Hz filter and a Notch filter of 50 Hz was used to discard alternate current contamination.

Six sleep−wake states were determined based upon combination of the dynamics of 5 EEG frequency domains (δ, 0.4−4 Hz; θ, 4.2−8 Hz; α, 8.2−12 Hz; σ, 12.2−14 Hz; β, 14.2−30 Hz) plus the integrated EMG, EOG, and body activity levels. These sleep−wake states were classified as active wake; passive wake; intermediate stage (preREM transients); light sleep and deep sleep and rapid eye movement sleep (REM).

Haloperidol-Induced Catalepsy. *Mouse.* Male mice (Balb/c) (Charles River; 20−23 g; fasted overnight) were randomly assigned to treatment groups and behavioral testing was performed blind to treatment. In the dose−response experiment, mice were injected with haloperidol (1.0 mg/kg, s.c.) and 0.5 h later given an oral dose of either vehicle, JNJ-40255293 (0.10, 1.0, 3.0, or 10 mg/kg), or, as a positive control, JNJ-27631734 (10 mg/kg). One hour later, catalepsy was measured by placing the forepaws of the mice on a horizontal bar elevated 3.5 cm above the bench for a maximum of 1 min. The duration of catalepsy was measured as the time before which the mouse either placed its forepaws on the bench or climbed onto the bar with all of its limbs. If the mouse remained cataleptic throughout the trial, the duration was recorded as 60 s. An additional study, catalepsy time was measured repeatedly in haloperidol (1.0 mg/kg, s.c.)-treated mice 0.5, 1, 2, and 4 h after oral administration of JNJ-40255293 (10 mg/kg), vehicle or the positive control JNJ-27631734 (10 mg/kg).

Rat. Haloperidol (0.63 mg/kg s.c.)-induced catalepsy was scored (0, 1, 2 or 3) by two independent observers at hourly intervals over a period of 4 h after challenge with haloperidol (0.63 mg/kg, s.c.) in male Wistar rats (Crl:WI; Charles River, Sulzfeld, Germany; 200−260 g; fasted overnight) that were cotreated with test compound or vehicle immediately prior to the haloperidol injection. The sum of the scores of both observers was used for further evaluation. Criteria for drug-induced reversal of haloperidol-induced catalepsy: scores of <4, <6, and <6 at 2, 3, and 4 h, respectively (3.5%, 5.1%, and 5.9% false positives, respectively, in a population of >250 control rats). Based on the fraction of responders to these criteria per dose level, ED_{50} values and corresponding 95% confidence limits were determined as indicated below (section Statistics).

Haloperidol-Induced Hypolocomotion. *Mouse.* Haloperidol (0.31 mg/kg, i.v.)-induced hypolocomotion was evaluated over a 30 min period starting immediately after the haloperidol challenge in male NMRI mice (Charles River, Sulzfeld, Germany; 20−26 g; fasted overnight) pretreated at a specified time interval with test compound or vehicle. Total distance traveled was measured in open field arenas (gray PVC cylinder with a height of 40 cm and a diameter of 22.5 cm) using the Noldus Ethovision XT Video Tracking System (version 3.1; Noldus, Wageningen, The Netherlands). Criterion for drug-induced reversal of the haloperidol-induced hypolocomotion: total distance > 2000 cm (5.3% false positives in controls; n > 500). Based on the fraction of responders to these criteria per dose level, ED_{50} values and corresponding 95% confidence limits were determined as indicated below (see Statistics subsection).

Rat. Male Wistar rats (Crl:WI; Charles River, Sulzfeld, Germany; 200−260 g; fasted overnight) were pretreated with test compound or solvent (10 mL/kg) and placed in individual cages. At a specified interval later, the rats were challenged with haloperidol (0.16 mg/kg, i.v.), and, immediately thereafter, motor activity was measured over a 30 min period in microprocessor-based motor activity cages (length × width × height: 43.5 × 43.5 × 41.5 cm^3; MED Associates, St. Albans, VT). The following all-or-none criteria were adopted for drug-induced effects: (1) reversal of hypolocomotion (>1500 counts; 5.9% false positives in 68 control rats), (2) induction of hyperlocomotion (>4500 counts; 0.0% false positives). Based on the fraction of responders to these criteria per dose level, ED_{50} values and corresponding 95% confidence limits were determined as indicated below (see Statistics subsection).

Haloperidol-Induced Impairment of Conditioned Avoidance Response in Rats. Male Wistar rats (Crl:WI; Charles River, Sulzfeld, Germany; 200−260 g; fasted overnight) were housed in individual cages provided with bedding material. The rats received five training sessions at 15 min intervals over a 1 h period to learn to avoid an electric shock when put in a "jumping box" (length × width × height: 30 × 30 × 30 cm^3) with an open top and a grid floor made of 15 pairs of iron bars (2 mm diameter; 6 mm interbar distance) that could be electrified with a source of alternative current (1.0 mA; Coulbourn Instruments Solid State Shocker/Distributor), which could be interrupted by a switch. During the training sessions, the rat was placed on the nonelectrified grid floor and the grid was electrified 10 s later for not more than 30 s, if the rat did not jump out of the box. Only rats that showed correct avoidance responses in all the last three training sessions were included for further experiments. Immediately after the last training section, selected rats were challenged with haloperidol (0.63 mg/kg, s.c.) and received simultaneously cotreatment with test compound or solvent. Subsequently, the rats were tested three times, that is, at 60, 90, and 120 min after the injection of test compound or solvent. Latency to avoidance was recorded. The median avoidance response over the three experimental sessions for each rat was used for further calculations. A median avoidance latency < 9 s was adopted as all-or-none criterion for reversal of the haloperidol-induced impairment of avoidance responding (occurring in only 2 out of 83 solvent-treated control rats). Based on the fraction of responders to the criteria adopted for drug-induced effects per dose level, ED50 values and corresponding 95% confidence limits were determined as indicated below (see Statistics subsection).

Apomorphine-Induced Agitation. Apomorphine (1.0 mg/kg, i.v.)-induced agitation (sniffing, licking, chewing) was scored (0, 1, 2 or 3) in male Wistar rats (Crl:WI; Charles River, Sulzfeld, Germany; 200−260 g; fasted overnight) at 5 min intervals for the subsequent 1 h period. Criterion for drug-induced stimulation of agitation: more than 8 scores of 3 (0.7% false positives), more than 9 scores of ≥2 (0.2% false positives), or more than 11 scores of ≥1 (0.03% false positives). Based on the fraction of responders to the criteria adopted for drug-induced effects per dose level, ED_{50} values and corresponding 95% confidence limits were determined as indicated below (see Statistics subsection). The time and dose of peak effect and onset and duration of action were interpolated from the nonlinear regression lines (polynomial second order function $y = a + bx + cx^2$) relating ED_{50} to time after administration, using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Onset and duration of action were interpolated at 4 times the peak-effect dose.

Reserpine-Induced Akinesia. Locomotor activity was measured in male CF-1 mice (Charles River Breeding Laboratories; 29−40 g; fasted overnight) and male Wistar rats (Crl:WI; Charles River Breeding Laboratories; 210−245 g; fasted overnight) in open field activity boxes (length, 45 cm; width, 45 cm; height, 30 cm; MED Associates, St. Albans, VT), each containing 20 equally spaced pairs of horizontal infrared photocell beams along one axis using automatic activity analysis software. One activity count corresponded to the consecutive interruption of two infrared beams placed 2.5 cm apart and 2 cm above the cage floor, and total distance traveled was quantified in centimeters.

Locomotor activity was measured for 1 h after oral administration of JNJ-40255293 or the positive control JNJ-27631734 (10 mg/kg) to either mice or rats that had been pretreated with reserpine (0.6 mg/kg, s.c.) 18 h earlier. Animals were randomly assigned to treatment groups.

Lesions of the Nigrostriatal Pathway. Unilateral lesions of the nigrostriatal pathway were produced by microinjecting 6-hydroxydopamine (6-OHDA; 12 μg) into the medial forebrain bundle of male Sprague−Dawley rats (Charles River Breeding Laboratories; 225−250 g; fasted overnight). Three weeks later, the rats were challenged with dopaminergic agents and their circling behavior was measured using an optical sensor connected to an automated computerized system, with one rotation being defined as a 360° turn. The lesion was validated based upon unilateral circling behaviors produced by the postsynaptic dopaminergic agonist apomorphine (0.05 mg/kg s.c.) and 2 weeks later by L-DOPA (10 mg/kg p.o. coadministered with 2.5 mg/kg p.o. carbidopa). Having pharmacologically confirmed the unilateral lesions, rats were dosed p.o. with either vehicle (20% hydroxypropyl-β-cyclodextrin) or JNJ-40255293 (0.1−10 mg/kg p.o.) and then 1 h later received either vehicle (20% hydroxypropyl-β-cyclodextrin) or L-DOPA (10 mg/kg p.o. plus 2.5 mg/kg p.o. carbidopa). A within-subject design was used allowing at least 2 days between drug-testing sessions.

Pentylenetetrazole Proconvulsant Assay. Male Wistar rats (Crl:WI; Charles River, Sulzfeld Germany; fasted overnight; 100−150 g) were given either vehicle (10% hydroxypropyl-β-dextrin p.o.) or 10

mg/kg JNJ-40255293 1 h prior to the i.v. administration of either 5, 10, 20, 40, 80, or 160 mg/kg pentylenetetrazole (n = 5/group). Following the administration of pentylenetetrazole, tremors and clonic and tonic seizures (of the fore and hind paws) were scored according to the following scale: 0 = absent; 1 = weak and delayed; 2 = weak but immediate; 3 = pronounced but delayed; 4 = pronounced and immediate. In addition, animals were observed for the 1 h period prior to dosing with pentylenetetrazole for the appearance to spontaneous (i.e., nonpentylenetetrazole-induced) convulsant activity. The ED_{50} values of pentylenetetrazole for inducing tremors, clonic convulsions, tonic convulsions of the fore paws, and tonic convulsions of the hind paws were calculated (see Statistics subsection) in the absence and presence of JNJ-40255293. A decrease or increase in the pentylenetetrazole ED_{50} with nonoverlapping 95% confidence intervals is considered to reflect a pro- or anticonvulsant effect, respectively. No difference in ED_{50} (overlapping 95% confidence intervals) indicates that, in this particular assay, a compound has no effect on the seizure threshold.

Drug Discrimination. *Training.* Drug discrimination assays were performed essentially as described elsewhere.[47] Male Sprague–Dawley rats (Charles Rivers, Germany), weighing 280 ± 20 g at the start of the experiment, were allowed ad libitum water access. Food access, however, was restricted to 9 g on Monday through Thursday, 13 g on Friday and Saturday, and 10 g on Sunday. This procedure allowed rats to maintain body weight and sustain a slow rate of weight gain until they reached a mature weight of approximately 425 g. The rats were trained to lever-press for a 45-mg food pellet on a fixed ratio 10 (FR-10) schedule such that prior treatment with the training drug or physiological saline produced responding on the "drug lever" (DL) or the "saline lever" (SL), respectively. Animals were trained to discriminate the following drugs: D-amphetamine (0.8 mg/kg s.c.), chlordiazepoxide (5 mg/kg i.p.), cocaine (10 mg/kg s.c.), 2,5-dimethoxy-4-methylamphetamine (DOM; 0.63 mg/kg i.p.), ethanol (1.5 g/kg i.p.), fentanyl (0.02 mg/kg s.c.), lysergic acid diethylamide (LSD; 0.16 mg/kg i.p.), or nicotine (0.6 mg/kg s.c.). Drugs were administered either 15 min (chlordiazepoxide, DOM, ethanol, LSD, nicotine) or 30 min (D-amphetamine, cocaine, fentanyl) before being placed into the operant chambers (MedAssociates, St. Albans, VT). Each session lasted for 15 min, and all the responses on both levers were recorded. At the beginning of each session, the fixed ratio to first pellet (FRF) value was noted. The FRF value was defined as the sum of the total number of responses on both levers until 10 responses had been made on the appropriate lever and the first reinforcement had been obtained. Discrimination training proceeded for each rat individually until 10 consecutive sessions occurred in which an FRF-value ≤ 14 was obtained. Animals reaching this criterion were used for testing. Having achieved criterion, animals were maintained on a 5 days/week schedule of daily drug (D) or saline (S) injections given in alternating sequences (e.g., D-S-S-D-S, S-D-D–S-S, S-D-S-D-D, D-S-D-S-D).

Testing. Test sessions in which JNJ-40255293 was given alone (generalization studies) or prior to the training drug (antagonism studies) were routinely conducted on Fridays. A record was made on which of the two levers the animal first made a total of 10 responses. This lever is referred to as the selected lever. Once this lever selection was established, the rat obtained the first food pellet and subsequent reinforcement was contingent upon pressing (FR-10) the selected lever. Testing was postponed to the next test day if the FRF-value had exceeded 14 on any of the most recent training days. The response rate (i.e., the total sum of the responses on both the DL and SL during the 15 min session) and the percentage responding on the selected lever (i.e., the ratio of the number of responses on the appropriate lever to the response rate) were calculated.

Statistics. *Determination of ED_{50} Values.* The percentage of receptor occupancy was plotted against dosage and the sigmoidal log dose–effect curve of best fit was calculated by nonlinear regression analysis, using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). From these dose–response curves, the graded ED_{50}'s (the dose producing 50% occupancy) with their 95% confidence limits was calculated. Graded ED_{50}'s were also calculated for the reversal of haloperidol-induced catalepsy in mice. For other in vivo studies (apomorphine-induced agitation, haloperidol-induced catalepsy in rats, haloperidol-induced conditioned avoidance response impairment; haloperidol-induced hypolocomotion in mice and rats, pentylenetetrazole-induced convulsions), categorical ED_{50}'s were calculated. All-or-none criteria for drug-induced effects were defined by analyzing a frequency distribution of a series of historical control data, aiming for less than 5% responders in the control population. The fraction of animals responding to these criteria in animals pretreated with test compound was determined per dose level ($n \geq 5$ in the relevant doses range; at least 3 dose levels). The categorical ED_{50} (the dose producing 50% responders to criterion) and corresponding 95% confidence limits were determined according to the modified Spearman-Kaerber estimate, using theoretical probabilities instead of empirical ones. This modification allows the determination of the ED_{50} and its confidence interval as a function of the slope of the log dose–response curve.[48]

In the sleep–wake EEG paradigm, a mixed-model ANOVA was used to analyze the changes in time spent in vigilance states and related variables. All data were presented as the means ± SEM.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Table 1: Inhibition of radioligand binding at a variety of receptors and transporters by JNJ-40255293 at 10 μM. Supplementary Figure 1: Reversal of haloperidol-induced hypolocomotion in rats 1 h after oral administration of JNJ-40255293, istradefylline, and ASP-5854. Supplementary Figure 2: Reversal of haloperidol-induced hypolocomotion in mice 0.5 h after oral administration of JNJ-40255293, istradefylline, and ASP-5854. Supplementary Figure 3: Reversal of haloperidol-induced conditioned avoidance impairment in rats 1 h after oral administration of JNJ-40255293, istradefylline, and ASP-5854. Supplementary Figure 4: Potentiation of apomorphine-induced agitation in rats 1 h after oral administration of JNJ-40255293, istradefylline. and ASP-5854. This material is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

Corresponding Author

*Mailing Address: Janssen Research and Development, PO Box 776, Welsh and McKean Roads, Spring House, PA 19477, United States. Phone: 215-628-7047. E-mail: bshook@its.jnj.com.

Author Contributions

Participated in research design: S.R., K.R., B.C.S., A.A.H.P.M., W.H.D., A.A., X.L., B.H., and J.R.A.. Conducted experiments: S.R., A.A., B.H., P.D.H., P.t.R., H.H., N.A., K.H., A.W., and J.V.. Contributed new reagents or analytic tools: B.C.S. and P.J.F.. Performed data analysis: S.R., K.R., A.A.H.P.M., W.H.D., A.A., X.L., B.R., J.R.A., and P.D.H.. Wrote or contributed to the writing of the manuscript: J.R.A., A.A.H.P.M., B.C.S., A.A., and W.H.D.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank Mrs. Eva Huybrechts and colleagues (K15 BVBA; Zoersel, Belgium; info@k15.be) for adapting the figures to the Journal requirements.

■ ABBREVIATIONS

6-OHDA, 6-hydroxydopamine; AB-MECA, (2*S*,3*S*,4*R*,5*R*)-5-[6-({[4-amino-3-(^{125}I)iodophenyl]methyl}amino)-9H-purin-9-yl]-3,4-dihydroxy-*N*-methyloxolane-2-carboxamide; ASP5854, 5-[5-amino-3-(4-fluorophenyl) pyrazin-2-yl]-1-isopropylpyridine-2(1H)-one; AW, active wake; cAMP, cyclic adenosine mono-

phosphate; BIIB014, 3-[(4-amino-3-methylphenyl)methyl]-7-(furan-2-yl)-3H-1,2,3-triazolo[4,5- d]pyrimidin-5-amine (V2006, vipadenant); CGS21680, 3-[4-[2-[[6-amino-9-[(2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]-purin-2-yl]amino]ethyl]phenyl]propanoic acid; CHO, Chinese hamster ovary; CNS, central nervous system; CPA, N^6-cyclopentyladenosine; CRE, cAMP-response element; CSF, cerebrospinal fluid; DOM, 2,5-dimethoxy-4-methylamphetamine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EEG, encephalography; EMG, electromyography; EOG, electrooculography; HPLC, high-pressure liquid chromatography; JNJ-27631734, 2-amino-4-phenyl-8-(pyrrolidin-1-ylmethyl)-5*H*-indeno[1,2-*d*]pyrimidin-5-one; JNJ-40255293, 2-amino-8-[2-(4-morpholinyl)ethoxy]-4-phenyl-5H-indeno[1,2-*d*]-pyrimidin-5-one; L-DOPA, L-dihydroxyphenylalanine; FDA, Food and Drug Administration; FR, fixed ratio; FRF, fixed ratio to first pellet; HEK, human embryonic kidney; LC, liquid chromatography; LSD, lysergic acid diethylamide; MED, minimum effective dose; MS, mass spectrometry; MSX-3, 3-(3-hydroxypropyl)-8-(3-methoxystyryl)-7-methyl-1-propargylxanthin phosphate disodium salt; N/A, not assayed; NAC, nucleus accumbens; NC, not computed; PD, Parkinson's disease; p.o., orally (per os); PFC, prefrontal cortex; PW, passive wake; REM, rapid eye movement; s.c., subcutaneously; SCH-58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo [1,5-*c*] pyrimidine; SCH-420814, 2-(2-furanyl)-7-[2-[4-[4-(2-methoxyethoxy)phenyl]-1-piperazinyl]ethyl]7H-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine-5-amine; SCH442416, 2-furan-2-yl-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-*e*]-[1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine; SEM, standard error of the mean; ST1535, 2-*n*-butyl-9-methyl-8-[1,2,3]triazol-2-yl-9H-purin-6-ylamine; SYN115, 4-hydroxy-4-methylpiperidine-1-carboxylic acid-(4-methoxy-7-morpholin-4-yl-benzothiazol-2-yl)-amide; TMSX, 7-methyl-(11)C]-(E)-8-(3,4,5-trimethoxystyryl)-1,3,7-trimethylxanthine; US, United States of America; ZM-241385, 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-*a*]-[1,3,5]triazin- 5-yl amino]ethyl) phenol

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