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Yawning and Hypothermia in Rats: Effects of Dopamine D3 and D2 Agonists and Antagonists

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Abstract

Rationale—Identification of behaviors specifically mediated by the dopamine D2 and D3 receptors would allow for the determination of *in vivo* receptor selectivity and aide the development of novel therapeutics for dopamine-related diseases.

Objectives—These studies were aimed at evaluating the specific receptors involved in the mediation of D2/D3 agonist-induced yawning and hypothermia.

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Methods—The relative potencies of a series of D2-like agonists to produce yawning and hypothermia were determined. The ability of D3- and D2-selective antagonists to inhibit the induction of yawning and hypothermia were assessed, and a series of D2/D3 antagonists were characterized with respect to their ability to alter yawning induced by a low and high dose of PD-128,907 as well as sumanirole-induced hypothermia.

Results—D3-preferring agonists induced yawning at lower doses than those required to induce hypothermia, and the D2-preferring agonist, sumanirole, induced hypothermia at lower doses than were necessary to induce yawning. The rank order of D3 selectivity was pramipexole > PD-128,907 = 7-OH-DPAT = quinpirole = quinelorane > apomorphine = U91356A. Sumanirole had only D2 agonist effects. PG01037, SB-277011A and U99194 were all D3-selective antagonists, whereas haloperidol and L-741,626 were D2-selective antagonists and nafadotride's profile of action was more similar to the D2 antagonists than to the D3 antagonists.

Conclusions—D3 and D2 receptors have specific roles in the mediation of yawning and hypothermia, respectively, and the analysis of these effects allow inferences to be made regarding the selectivity of D2/D3 agonists and antagonists with respect to their actions at D2 and D3 receptors.

Keywords

D2 receptors; D3 receptors; yawning; hypothermia; pramipexole; quinpirole; PG01037; SB-277011A; agonists; antagonists

Introduction

Dopamine D2 and D3 receptors are both members of the D2-like family of dopamine receptors, and are known to possess a high degree of sequence homology (52% overall and 75% in the transmembrane domains; Sokoloff et al. 1990), and a partially overlapping pattern of distribution in the brain. For example, D2 receptors are expressed at relatively high levels within cortical, as well as limbic regions, while the D3 receptor has been shown to possess a much more restricted limbic pattern of distribution in both the rat (Levesque et al. 1992) and human brain (Gurevich and Joyce 1999). These high levels of expression within limbic brain regions have led many to hypothesize that the D2 and D3 receptors are of particular interest as pharmacologic targets for the treatment of a variety of movement and psychiatric disorders including Parkinson's disease, restless leg syndrome, depression, and schizophrenia (e.g., Joyce 2001; Happe and Trenkwalkder 2004), as well as a variety of aspects of drug abuse (e.g., Heidbreder et al. 2005; Newman et al. 2005). Due in part to the lack of highly selective agonists and antagonists, the receptor(s) mediating either the therapeutic or mechanistic effects are yet to be fully elucidated.

Although several agonists and antagonists have been reported to be over 100-fold selective for either the D3 (e.g., Stemp et al. 2000; Grundt et al. 2005) or D2 (e.g., Vangveravong et al. 2006) receptors based on *in vitro* binding studies, a large degree of variability exists with respect to the reported *in vitro* binding affinities and D2/D3 selectivity ratios. A variety of factors may account for these differences in affinity and selectivity including differences in receptor species, expression systems, radioligands, and/or assay conditions. For example, reported binding affinities for pramipexole at the D2 receptor range from 3.9 nM to 955 nM depending upon whether agonist or antagonist radioligands were used (Mierau et al. 1995; Millan et al. 2002) while reported D3 selectivity ratios range from 2- to 488-fold selective for the D3 over D2 receptor depending upon whether binding affinities from cloned human receptor cell systems or human brain tissue are used to make the determinations (Seeman et al. 2005; Gerlach et al. 2003). Furthermore, *in vitro* binding studies often provide greater affinity and selectivity values than those obtained through functional studies suggesting that

differences in D2 and D3 efficacy may also greatly influence a ligand's receptor selectivity. For example, in three separate studies which characterized D2/D3 agonists based on their binding affinities for the D2 and D3 receptors and ability to stimulate mitogenic activity, quinpirole was found to be either 9-, 15- or 36-fold selective for the D3 over D2 receptor as determined by radioligand binding, but the D3 selectivity ratios for quinpirole dropped to 2.5-, 1.3- and 3.3-fold when ED_{50} values for the induction of mitogenic activity were compared (Pugsley et al. 1995; Chio et al. 1994; Sautel et al. 1995).

The identification of agonists and antagonists highly selective for the D2 and/or D3 receptors has been complicated by a lack of well characterized behavioral effects specifically mediated by either the D2 or D3 receptor. While D2/D3 agonists have been shown to modulate body temperature, locomotor activity, and certain neuroendocrine responses in addition to other behavioral measures (Faunt & Crocker 1987; Millan et al. 1995; Depoortere et al. 1996; Smith et al. 1997; Boulay et al. 1999a), few of these effects have been fully characterized and well validated. There is strong pharmacological and genetic evidence in support of subtype selective *in vivo* effects for the induction of hypothermia resulting from D2 receptor activation, and significant pharmacological evidence for the induction of yawning resulting from agonist activation of the D3 receptor.

The first indication that D2/D3 agonist-induced hypothermia was mediated by the D2 but not D3 receptor was the finding that D3 receptor-deficient mice displayed a normal hypothermic response to D2/D3 agonists while the effect was completely absent in D2 receptor-deficient mice (Boulay et al. 1999a; Boulay et al. 1999b). This was later supported by pharmacologic studies in rats that demonstrated that the D2-preferring antagonist, L-741,626, produced a dose-dependent inhibition of D2/D3 agonist-induced hypothermia, whereas the D3-preferring antagonist A-437203 failed to alter the hypothermic response at any dose tested (Chaperon et al. 2003).

Yawning behavior in rats has been a long studied phenomenon, and is known to be regulated by a variety of neurotransmitter systems including cholinergic (Urba-Holmgren et al. 1977; Yamada & Furkawa 1980), serotonergic (Stancampiano et al. 1994), and dopaminergic (Mogilnicka & Klimek 1977; Holmgren & Urba-Holmgren 1980) systems associated with the paraventricular nucleus of the hypothalamus (Argiolas & Melis 1998). Recently, a specific role for the D3 receptor in the induction of yawning behavior has also been demonstrated. A series of D3-preferring agonists induced dose-dependent increases in yawning behavior over low doses, with inhibition of yawning occurring at higher doses resulting in a characteristic inverted U-shaped dose-response curve. Several D3-preferring antagonists were also shown to selectively inhibit the induction of yawning behavior, while the D2-preferring antagonist, L-741,626, produced a selective rightward and upward shift in descending limb of the dose-response curve for D2/D3 agonist-induced yawning (Collins et al. 2005). Thus, although it has been suggested that the induction of yawning is mediated by activation of the D2 receptor (Millan et al. 2000), our data indicated that the induction of yawning by D2/D3 agonists is mediated by a selective activation of the D3 receptor while inhibition of yawning behavior at higher doses is a result of a concomitant D2 receptor activation.

The present studies were aimed at further characterizing the roles of the D2 and D3 receptors in the regulation of body temperature and yawning behavior. Thus, a series of D2-like agonists with a range of reported *in vitro* selectivities for the D3 over D2 receptor (pramipexole ≥ PD-128,907 = 7-OH-DPAT > quinpirole = quinelorane >apomorphine > U91356A > sumanirole), as well as two D4-preferring agonists (ABT-724 and PD-168,077) were assessed for their ability to induce yawning and hypothermia, while a series of D2/D3 antagonists with a similar range of reported in vitro selectivities (PG01037 = SB-277011A

>> U99194 > nafadotride > haloperidol > L-741,626) were characterized for their ability to modulate the induction of yawning and hypothermia in the rat. Convergent evidence support the hypotheses that the induction of hypothermia and yawning behavior are mediated by the selective activation of the D2 and D3 receptors. Furthermore, these studies suggest that the minimal effective dose (M.E.D.) for the induction and inhibition of yawning behavior and hypothermia may provide a means for the determination of *in vivo* D3 and D2 receptor potency measures for agonists and antagonists respectively.

Methods

Subjects

Male Sprague-Dawley rats weighing 250–300 g were obtained from Harlan (Indianapolis, IN) and given free access to standard Purina rodent chow and water. Rats were housed three to a cage for all yawning studies, and singly housed for hypothermia studies. All rats were maintained in a temperature (21–23 °C) and humidity controlled environment, on a 12-h dark/light cycle with lights on at 7:00 AM. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health, and all experimental procedures were approved by the University of Michigan Committee on the Use and Care of Animals.

Observation of Yawning Behavior

Yawning behavior was defined as a prolonged (~1 sec.), wide opening of the mouth followed by a rapid closure. On the day of testing, rats were transferred from their home cage to a test chamber (48 cm × 23 cm × 20 cm clear rodent cage with standard cob bedding), and allowed to habituate to the chamber for a period of 30 min. A sterile water injection was administered 30 min prior to the injection of agonist or vehicle; behavioral observations began 10 min thereafter, and yawns were scored for a period of 20 min. A mirror was placed behind two stacked observation cages to allow for the simultaneous observation of two rats by a trained observer. Each rat was tested multiple times with at least 48 hrs between test sessions to allow for drug washout. Food and water were unavailable during test sessions, and all experiments were conducted between the hours of 12:00 PM and 6:00 PM. Yawning induced by peak doses of agonists were redetermined throughout the duration of the experiment to insure there were no changes in agonist-induced yawning behavior.

Measurement of Core Body Temperature

Rats were anesthetized with ketamine (100 mg/kg; i.m.) and xylazine (10 mg/kg; i.m.) and their abdominal area was shaved and cleaned with iodine swabs prior to surgical implantation of radio-telemetric probes (E-4000 E-Mitter, Mini-Mitter, Bend, OR, USA). A small rostral-caudal incision was made in the abdominal wall to allow for insertion of the probe, and the abdominal wall was closed using absorbable, 5-0 chromic gut suture, and the skin was closed using 5-0 Ethilon® suture. Rats were allowed at least 5 days to recover prior to the beginning of experimentation.

On the day of testing, rats were weighed and returned to their cages which were placed onto a receiving pad (ER-4000 Receiver, Mini-mitter, Bend, OR) to allow for the real time detection and recording of core body temperature. Temperature measurements were taken every min with at least 45 min of baseline temperature data recorded prior to the administration of antagonist or vehicle. Agonist or vehicle injections were administered 30 min after either antagonist or vehicle pretreatments, and core body temperature was recorded for a period of 120 min thereafter. Rats were removed from the receivers for a period of 5 min to allow for injections to be administered, but were otherwise uninterrupted. Each rat

was tested multiple times with each dose of one agonist with at least a 48 hr drug washout period allowed between test sessions. All experiments were carried out between the hours of 9:00 AM and 3:00 PM.

D2-Like Agonist-Induced Yawning and Hypothermia

A series of D2-like agonists were assessed for their ability to induce yawning behavior and hypothermia in rats. The following agonists were assessed at 1/2 log unit dose increments: 7-OH-DPAT (0.0032 – 1.0 mg/kg), ABT-724 (0.001 – 1.0 mg/kg), apomorphine (0.001 – 1.0 mg/kg), PD-128,907 (0.0032 – 1.0 mg/kg), PD-168,077 (0.0032 – 1.0 mg/kg), pramipexole (0.0032 – 3.2 mg/kg), quinelorane (0.0001 – 0.032 mg/kg), quinpirole (0.0032 – 1.0 mg/kg), sumanirole (0.032 – 3.2 mg/kg), and U91356A (0.032 – 1.0 mg/kg). Yawning and hypothermia were determined in separate groups of rats, with subgroups of rats receiving each dose of an agonist in random order.

Effects of D2-like Antagonists on Hypothermia and Yawning Behavior

The ability of the D2 antagonist, L-741,626, and the D3 antagonist, U99194, to alter hypothermia induced by either D2/D3 agonists, or 8-OH-DPAT was investigated in separate groups of rats for each agonist. Pretreatments of 1.0 mg/kg L-741,626, 3.2 mg/kg U99194, or vehicle were presented in random order, while the agonist dose (0.1 mg/kg 7-OH-DPAT, 1.0 mg/kg 8-OH-DPAT, 0.1 mg/kg apomorphine, 0.32 mg/kg PD-128,907, 0.32 mg/kg pramipexole, 0.01 mg/kg quinelorane, 0.1 mg/kg quinpirole, 1.0 mg/kg sumanirole and 0.32 mg/kg U91356A) remained constant.

The D2 antagonist, L-741,626, and the D3 antagonist, PG01037, were assessed for their ability to alter D2/D3 agonist-induced yawning in separate groups of rats for each agonist. Each rat was tested six times, with pretreatments of either 1.0 mg/kg L-741,626, 32.0 mg/kg PG01037, or vehicle presented in random order prior to each of two doses of a single agonist (0.032 and 0.1 mg/kg 7-OH-DPAT, 0.032 and 0.1 mg/kg apomorphine, 0.1 and 0.32 mg/kg PD-128,907, 0.1 and 0.32 mg/kg pramipexole, 0.0032 and 0.01 mg/kg quinelorane, 0.032 and 0.1 mg/kg quinpirole, 3.2 mg/kg sumanirole, and 0.1 and 0.32 mg/kg U91356A).

The doses of agonists selected for the yawning study represent low doses that produce peak levels of yawning and high doses that are on the descending limb of the dose-response curves for yawning behavior. These high doses were also used in the hypothermia study as they all possess significant hypothermic effects. The doses for the antagonist were chosen based on their ability to selectively shift the ascending (PG01037 and U99194) or descending (L-741,626) limbs of the dose response curves for PD-128,907 induced yawning in rats (Collins et al. 2005).

Effects of D2/D3 Antagonists on PD-128,907-Induced Yawning Behavior and Sumanirole-Induced Hypothermia

A series of antagonists with varying *in vitro* selectivities for the D2 and D3 receptors were examined with regard to their ability to antagonize hypothermia induced by 1.0 mg/kg sumanirole, as well as yawning induced by 0.1 and 0.32 mg/kg of the D3-preferring agonist, PD-128,907. The D3-preferring antagonists nafadotride (0.1, 0.32, and 1.0 mg/kg), U99194 (1.0, 3.2, and 10.0 mg/kg), SB-277011A (3.2, 32.0, and 56.0 mg/kg), and PG01037 (3.2, 32.0, and 56.0 mg/kg), as well as the D2-preferring antagonists L-741,626 (0.32, 1.0, and 3.2 mg/kg) and haloperidol (0.01, 0.032, and 0.1 mg/kg), were given 30 min prior to the administration of either sumanirole in hypothermia studies or PD-128,907 in yawning studies. Separate groups of rats were used for yawning and hypothermia studies with subgroups of rats for each agonist. Doses were administered in random order.

Drugs

(±)-7-OH-DPAT, (−)-apomorphine, PD-128,907, quinelorane, and (−)-quinpirole were obtained from Sigma Chemical Co (St. Louis, Mo). L-741,626, PD-168,077, and U99194 were obtained from Tocris (Ellisville, MO). ABT-724 was prepared and generously provided by Dr. Kenner Rice (Chemical Biology Research Branch, NIDA, Bethesda, MD), PG01037 by Drs. Amy H. Newman and Peter Grundt (Medicinal Chemistry Section-NIDA, Baltimore, MD), pramipexole and SB-277011A by Drs. Jianyong Chen and Shaomeng Wang (University of Michigan, Ann Arbor, MI), and sumanirole by Drs. Cédric Chauvignac and Stephen Husbands (University of Bath, Bath, U.K.). U91356A was provided by Dr. Lisa Gold (Pfizer, Ann Arbor, MI). All drugs were dissolved in sterile water with the exception of L-741,626, which was dissolved in 5% ethanol with 1M HCl, PD-168,077 which was made up fresh daily, and dissolved in 5% ethanol, and PG01037 and SB-277011A, which were dissolved in 10% β-cyclodextrin. All drugs were administered subcutaneously (s.c.) in a volume of 1 ml/kg. The 56.0 mg/kg doses of SB-277011A and PG01037 were administered in a volume of 3 ml/kg s.c. due to solubility limitations.

Data Analysis

Determination of dose-response curves for agonist induced hypothermia were conducted with 6 rats per group with results expressed as the mean change in body temperature 30 min post agonist injection compared to the body temperature 1 min prior to the agonist injection ± standard error of the mean (SEM). All yawning studies were conducted with 8 rats per group with results expressed as mean number of yawns during the 20 min observation period ± SEM. A one-way, repeated-measures ANOVA with post-hoc Dunnett's tests were used to determine if agonist-induced yawning or hypothermia were significantly different from vehicle treated animals (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Significant differences in the maximal amount of yawning elicited by agonists were determined by one-way repeated-measures ANOVA with post-hoc Tukey's HSD tests. Significant effects of antagonists on the induction of yawning and hypothermia were determined by one-way, repeated-measures ANOVA with post-hoc Dunnett's tests.

The M.E.D. for D3 agonist activity ($M.E.D._{D3}$) was defined as the smallest dose that produced a statistically significant increase in yawning. The M.E.D. for D2 agonist activity ($M.E.D._{D2}$) was defined as the smallest dose that produced a statistically significant decrease in core body temperature. Selectivity ratios were calculated as the $M.E.D._{D2}$/ $M.E.D._{D3}$. Similar M.E.D. values were established for the antagonists ($M.E.D._{ANT.D2}$ and $M.E.D._{ANT.D3}$) and defined as the M.E.D. for inhibition of hypothermia or yawning induced by D2 and D3 agonists, respectively.

Results

Agonist-Induced Yawning Behavior and Hypothermia

As shown in Figure 1, seven of the eight agonists with significant affinity for the D3 and D2 receptors induced dose-dependent increases in yawning behavior over low doses, with inhibition of yawning and significant decreases in core body temperature observed at higher doses. With the exception of apomorphine and U91356A, there were no significant differences between the maximal amounts of yawning produced by these agonists, and they will subsequently be referred to as D3-preferring agonists. Unlike the D3-preferring agonists, the D2- and D4-preferring agonists differed in their ability to induce yawning and hypothermia in rats. As shown in Figure 1, sumanirole induced significant increases in yawning, although these increases were relatively small and observed only at the highest dose, whereas significant decreases in core body temperature were observed at lower doses; sumanirole will subsequently be referred to as a D2-preferring agonist. The D4-preferring

agonists, ABT-724 and PD-168,077 (Figure 2), failed to induce significant levels of yawning or hypothermia over a wide range of behaviorally active doses (Brioni et al. 2004; Enguehard-Gueiffier et al. 2006) suggesting that, at these doses, they are devoid of agonist activity at the D3 and D2 receptors.

Table 1 shows the $M.E.D._{D2}$ and $M.E.D._{D3}$ values, as well as the *in vivo* selectivity ratios for each of the agonists. The selectivity ratios obtained for the seven D3-preferring agonists, as calculated from the M.E.D.s for the induction of yawning and hypothermia, range from 3.2 to 32.0, indicating that these agonists were more potent at inducing yawning behavior than in producing hypothermia. Unlike the other D2/D3 agonists, the currently available *in vitro* data suggests that sumanirole preferentially binds the D2 over D3 receptor (Piercey et al. 1996; Heier et al. 1997), and in the current studies sumanirole displayed a distinctly different profile of activity. Not only was sumanirole more potent at inducing hypothermia than yawning, but as will be discussed later, the low levels of yawning produced by sumanirole may not be mediated through the D3 receptor, and therefore the $M.E.D._{D3}$ and D2/D3 ratio for sumanirole in Table 1 are placed in parentheses.

Antagonism of D2/D3 Agonist-Induced Yawning and Hypothermia

As shown in Table 2, the D3 antagonist PG01037 and the D2 antagonist L-741,626 produced differential effects on yawning behavior, and these effects were dependent on the dose of agonist tested. At a dose of 32.0 mg/kg, PG01037 significantly inhibited yawning induced by the low doses of all D3-preferring agonists, while having no effect on the low levels of yawning observed at the high doses of these agonists. Unlike with the D3-preferring agonists, the small amount of yawning produced by the D2-preferring agonist, sumanirole, was not significantly altered by administration of PG01037, but was completely blocked by the cholinergic antagonist, scopolamine (data not shown), suggesting that it may be mediated by cholinergic rather than by D3 receptors. Pretreatment with the D2 antagonist L-741,626 (1.0 mg/kg) did not significantly alter induction of yawning by low doses of D3-preferring agonists, but significantly increased yawning induced by high doses of all D2/D3 agonists, including sumanirole. This dose of L-741,626 was also found to significantly antagonize the induction of hypothermia induced by high doses of all D3-preferring agonists as well as the D2-preferring agonist, sumanirole (Table 3). Conversely, pretreatment with a behaviorally active dose of the D3 antagonist, U99194, did not significantly alter the induction of hypothermia resulting from any of the D2/D3 agonists tested (Table 3).

Antagonism of PD-128,907-Induced Yawning

The left two panels of Figure 3 show the effects of the D3-preferring antagonists on yawning induced by a low and high dose of the D3-preferring agonist PD-128,907. Pretreatment with all of the antagonists dose-dependently inhibited the induction of yawning by the low dose of PD-128,907 (left panel, Figure 3). Differences were observed, however, with respect to the effects of the antagonists on yawning induced by the high dose of PD-128,907. PG01037, SB-277011A, and U99194 had no effect on the low levels of yawning elicited by this high dose of PD-128,907, whereas pretreatment with the highest two doses of nafadotride resulted in significant increases in yawning induced by the high dose of PD-128,907 (center panel, Figure 3). The M.E.D. for the inhibition of yawning induced by 0.1 mg/kg PD-128,907 ($M.E.D._{D3\ ANT}$) for both PG01037 and SB-277011A was 32.0 mg/kg, while the $M.E.D._{D3\ ANT}$ for U99194 was 3.2 mg/kg, and 1.0 mg/kg for nafadotride (Table 1).

The two left panels of Figure 4 demonstrate that, similar to nafadotride, the D2-preferring antagonists, haloperidol and L-741,626, produced increases in the amount of yawning observed following administration of the high dose of PD-128,907 (center panel, Figure 4).

Moreover, these effects were observed at doses that did not alter yawning increased by the low dose of PD-128,907 (left panel, Figure 4); however decreases in yawning induced by this low dose of PD-128,907 were observed at higher doses for both of these antagonists. The M.E.D.$_{D3\ ANT}$ for L-741,626 and haloperidol were 3.2 and 0.1 mg/kg, respectively (Table 1).

Antagonism of Sumanirole Induced Hypothermia

The effects of the D3-preferring antagonists PG01037, SB-277011A, U99194 and nafadotride on sumanirole-induced hypothermia are shown in the right panel of figure 3. There were no significant effects of PG01037, SB-277011A or U99194 on the hypothermia produced by 1.0 mg/kg sumanirole. Larger doses of PG01037 and SB-277011A could not be given due to solubility limitations, and larger doses of U99194 were not used as they have been shown to produce anti-cholinergic effects (Goudie et al. 2001; Collins et al. 2005); for this reason, M.E.D.$_{D2\ ANT}$ values and D2/D3 ratios for these antagonists could not be calculated (Table 1). A significant and dose-dependent inhibition of sumanirole-induced hypothermia was observed following administration of nafadotride (right panel, Figure 3), with an M.E.D.$_{D2\ ANT}$ of 0.32 mg/kg (Table 1). Similarly, haloperidol and L-741,626 both produced a significant and dose-dependent inhibition of sumanirole-induced hypothermia (right panel, Figure 4), with M.E.D.$_{D2\ ANT}$ values of 0.032, and 1.0 mg/kg respectively (Table 1).

Discussion

The current studies replicate and extend the findings of a previous study that suggested that the induction of yawning by low doses of D2/D3 agonists is mediated by the selective activation of the D3 receptor, whereas the inhibition of yawning occurring at higher doses is mediated by a concomitant activation of the D2 receptor (Collins et al., 2005). As was demonstrated in the earlier paper, yawning induced by a low dose of the D3-preferring agonist PD-128,907 was selectively, and dose-dependently inhibited by the D3 antagonists, PG01037, SB-277011A, and U99194, whereas the inhibition of yawning observed at a high doses of PD-128,907 was reversed by the selective D2 antagonist L-741,626, but not PG01037, SB-277011A, nor U99194.

The current studies extend the previous findings in several ways. In addition to evaluation of agonist and antagonist interactions on yawning, the effects of the D2/D3 agonists alone and in combination with selective antagonists were evaluated on core body temperatures to test the notion that the hypothermic effects of these agonists are mediated by the activation of the D2, but not the D3 or D4 receptor (Boulay et al. 1999a; Boulay et al. 1999b; Chaperon et al. 2003). Several lines of evidence presented herein support this notion. The selective D2 agonist, sumanirole, produced decreases in body temperature at relatively low doses that did not induce yawning. The hypothermic effects of sumanirole were prevented by prior administration of the D2-preferring antagonists, haloperidol and L-741,626. L-741,626 also inhibited the hypothermic effects of high doses of all of the D3-preferring agonists in addition to producing dramatic increases in yawning when combined with the same high doses of D3-preferring agonists. The latter is likely to reflect reversal of the D2-mediated inhibition of yawning produced at high doses of the agonists, and is consistent with the notion that these antagonists are D2-selective and that the suppression of yawning and hypothermic effects observed at relatively high doses of D2/D3 agonists are D2 agonist-mediated effects. Importantly, these differential effects of D3 and D2 antagonists on yawning induced by low and high doses of D2/D3 agonists were observed with all of the D3-preferring agonists tested in the current study (Table 2), and occurred at doses of PG01037 that do not alter the induction of yawning by physostigmine or TFMPP (Collins et al. 2005), and a dose of L-741,626 that does not alter the induction of hypothermia by the

serotonin 1A agonist, 8-OH-DPAT (Table 2) suggesting that these effects are a result of a selective antagonist activity at D3 and D2 receptors, respectively.

These *in vivo* measures of selective D3 (yawning) and D2 (hypothermia) activation were used to characterize ten D2-like agonists and six D2/D3 antagonists. This extensive evaluation, comparing the potency of each agonist to produce increases in yawning with its potency to produce hypothermia (Table 1), indicated that pramipexole was the most selective D3 agonist, followed by PD-128,907, quinelorane, quinpirole and 7-OH-DPAT with nearly equal D3 selectivity. Both apomorphine and U91356A were relatively non selective D2/D3 agonists, inducing yawning at doses that were only slightly lower than those required to decrease body temperature. Sumanirole was a selective D2 agonist. Although sumanirole increased yawning slightly at doses that were higher than those necessary to decrease body temperature, this yawning was not sensitive to the D3-selective antagonist, PG01037, but was inhibited by the cholinergic antagonist scopolamine and may therefore represent cholinergic rather than D3 activation. McCall et al. (2005) reported a 200% increase in striatal acetylcholine release in rats at doses of sumanirole roughly equivalent to those which induced yawning. The two D4-preferring agonists, given at behaviorally active doses (Brioni et al. 2004; Enguehard-Gueiffier et al. 2006), did not produce either yawning or hypothermia suggesting that at these doses, they are devoid of significant D2 and D3 receptor agonist activity.

As was seen with the agonists, distinct behavioral profiles emerged for D3- and D2-preferring antagonists. Three of the four D3-preferring antagonists, PG01037, SB-277011A, and U99194 inhibited yawning at doses that did not alter hypothermia suggesting they function as selective D3 antagonists *in vivo*. The doses of these antagonists that were able to be tested was limited by solubility (PG01037 and SB-277011A) and anti-cholinergic activity (U99194), and thus *in vivo* D2/D3 selectivity ratios were indeterminate other than being slightly greater than 1. Interestingly, nafadotride, which is mildly D3-preferring *in vitro*, and generally considered to be a D3-preferring antagonist *in vivo* (e.g., Richtand et al. 2000; Leriche et al., 2003), displayed a profile of activity that was more like those of the D2 antagonists, haloperidol and L-741,626, than of the other D3-preferring antagonists. L-741,626, haloperidol and nafadotride were all more potent at inhibiting the induction of hypothermia and increasing high dose yawning, however, suppression of low dose yawning was also observed with each of these antagonists, and thus were all determined to be ~3-fold selective for the D2 over D3 receptor *in vivo*.

Evidence provided in the current, and past (Collins et al. 2005), studies support distinct roles for the D2 and D3 receptors mediating the hypothermic and yawning effects of D2/D3 agonists although these generalizations are contrary to earlier characterizations (see, Millan et al. 2000). These investigators determined that the hypothermic effects of 7-OH-DPAT were mediated by agonist activity at both the D2 and D3 receptor as it was attenuated by the D3 antagonists, S33084 and GR218231, as well as the D2 antagonist, L-741,626. Furthermore, they concluded that 7-OH-DPAT-induced yawning was mediated by the D2, but not D3 receptor as they observed inhibition of yawning with L-741,626, but not S33084 or GR218321. Although our data do not support this interpretation, we recognize that relatively large doses of D3-preferring agonists induce hypothermia, and likewise that relatively large doses of L-741,626 suppress yawning induced by D3-preferring agonists. However, these effects likely represent a loss of receptor selectivity rather than a primary effect of the agonists and antagonists, a notion that is supported by the biphasic nature of the D2/D3 agonists and antagonists with respect to their effects on yawning and hypothermia. In the current study, all D3-preferring agonists, including 7-OH-DPAT, induced yawning at low doses, with inhibition of yawning and induction of hypothermia occurring at higher, presumably less selective, doses. Similarly, at relatively low doses, L-741,626, haloperidol

and nafadotride equipotently increased high dose yawning and inhibited hypothermia, while inhibition of yawning induced by a low, presumably D3-selective, dose PD-128,907 was not observed until higher doses. Moreover, in the current study, the D3 antagonists PG01037, SB-277011A and U99194 all selectively inhibited PD-128,907-induced yawning while failing to alter the induction of hypothermia by sumanirole suggestive of a selective D3 antagonist activity.

While the MEDs for the inhibition of yawning by PG01037 and SB-277011A (32.0 mg/kg for both) are slightly higher than those reported for SB-277011A on a variety of operant behaviors (3.0 – 24 mg/kg; Andreoli et al. 2003; Di Ciano et al. 2003; Xi et al. 2004; Gilbert et al. 2005; Xi et al. 2005; Cervo et al. 2007) and are likewise higher than might be expected based on *in vitro* D3 affinities of 0.7 nM and 10.7 nM respectively (Stemp et al. 2000; Grundt et al. 2005) there is no evidence to suggest that the inhibition of yawning by these antagonists results from anything other than an antagonist activity at the D3 receptor. Not only did PG01037 and SB-277011A not inhibit sumanirole-induced hypothermia or increase yawning induced by high doses of PD-128,907 in the current studies at doses up to 56.0 mg/kg, but SB-277011A also failed to induce catalepsy and increases plasma prolactin levels at doses up to 78.8 and 93 mg/kg; p.o. respectively (Reavill et al. 2000). However, this is not to say that these antagonists are completely devoid of D2 antagonsit activity as U99194 has been reported to inhibit the induction of hypothermia with an ED_{50} of 12.9 mg/kg (Audinot et al. 1998) suggesting that inhibition of sumanirole-induced hypothermia by PG01037, SB-277011A and U99194 would have been observed if higher, less selective doses would have been assessed. Unequivocal resolution of these issues will depend on greater selectivity of ligands for these receptors.

The rank order of the *in vivo* D3 selectivity ratios obtained for these agonists and antagonists (Table 1) is in general agreement with similar determinations reported for *in vitro* binding studies. The magnitudes of the *in vivo* selectivities reported herein are much lower than those obtained by *in vitro* binding studies. However, similar differences have been reported when *in vitro* binding and functional assays are compared (Pugsley et al., 1995; Chio et al., 1994; Sautel et al., 1995), and are therefore not surprising. These data suggest that while comparisons of *in vitro* binding affinities provide an estimation of receptor selectivity, the utilization of *in vitro* functional assays and behavioral measures may provide a more accurate measure of an agonist or antagonist's selectivity as they allow for both potency and efficacy measures to be made, and may therefore be more informative in interpreting the *in vivo* pharmacology of D2-like agonists and antagonists.

To summarize, the results of these studies provide further support for specific roles for the D3 and D2 receptors in the mediation of D2/D3 agonist-induced yawning behavior and hypothermia, respectively, and demonstrate the usefulness of yawning and hypothermia in the characterization of *in vivo* D3 and D2 receptor activity. They are the first to provide *in vivo* determinations and comparisons of D3 receptor selectivities for a series of D2/D3 agonists with a range of *in vitro* selectivities for the D3 or D2 receptors. Thus, these data suggest that yawning and hypothermia may provide useful endpoints for the evaluation of *in vivo* antagonist activity and selectivity of future antagonists with improved solubility and selectivities for the D3 or D2 receptors.

Acknowledgments

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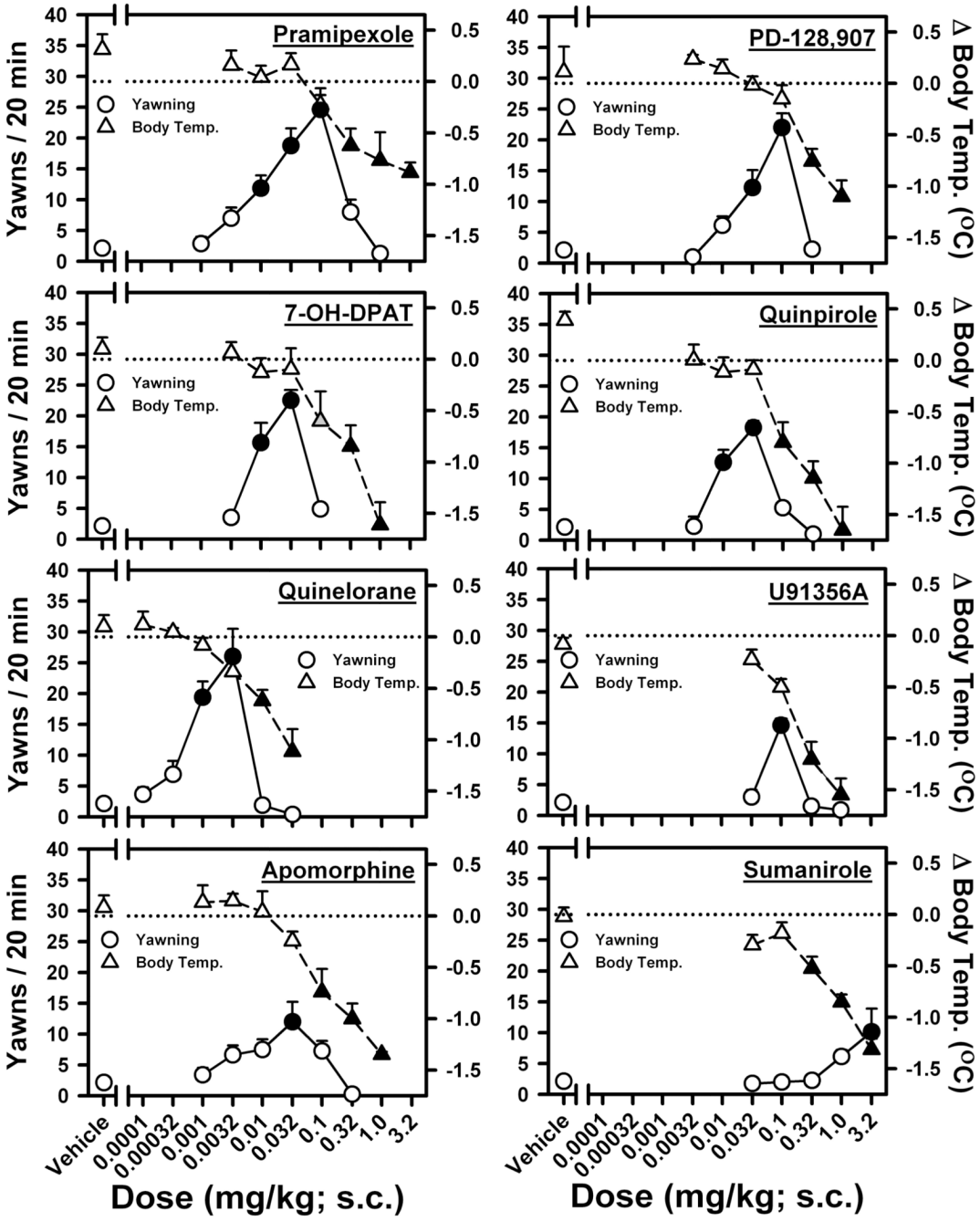

Figure 1.

Dose-response curves for D2/D3 agonist-induced yawning (○), and hypothermia (Δ). Characterization of pramipexole, PD-128,907, 7-OH-DPAT, quinpirole, quinelorane, U91356A, apomorphine, and sumanirole was conducted in different groups of rats, with data presented as mean (±SEM), n=8, number of yawns during a 20 minute observation period, and mean (±SEM), n=6, change in core body temperature as measured 30 min after, compared to 1 min before agonist injection. Gray filled, $p<0.05$, and black filled, $p<0.01$, symbols represent significant levels of yawning or hypothermia compared to vehicle treated rats as determined by one-way, repeated-measure ANOVA with post-hoc Dunnett's tests.

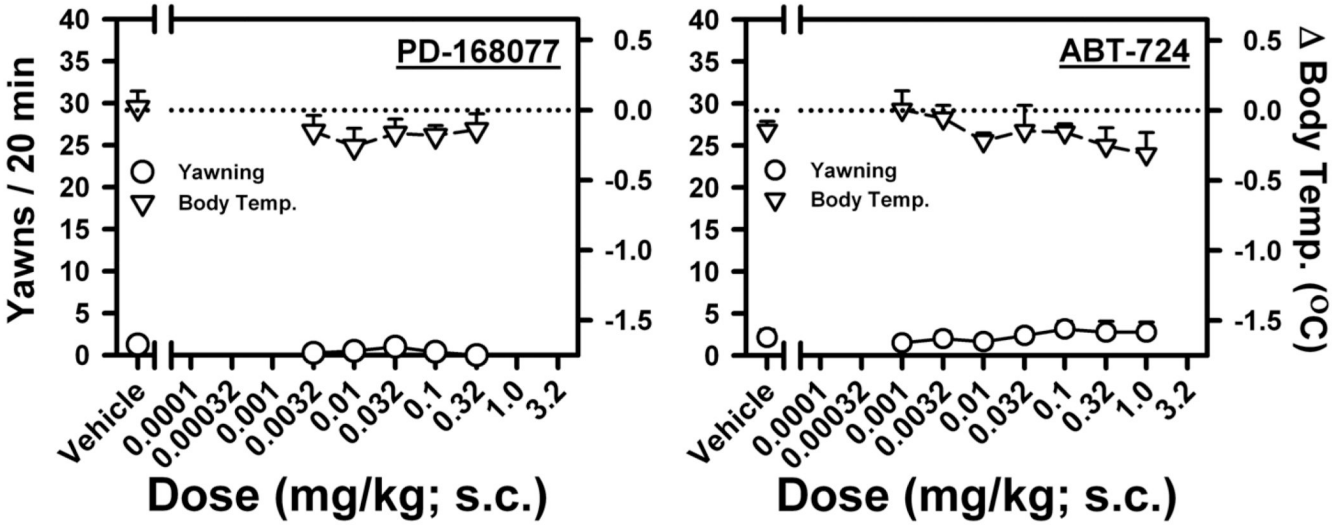

Figure 2.

Dose-response curves for D4-preferring agonist-induced yawning (○), and hypothermia (Δ). Characterization of ABT-724 and PD-168,077 was conducted in different groups of rats, with data presented as mean (±SEM), n=8, number of yawns during a 20 minute observation period, and mean (±SEM), n=6, change in core body temperature as measured 30 min after, compared to 1 min before agonist injection. Gray filled, $p<0.05$, and black filled symbols, $p<0.01$, represent significant levels of yawning or hypothermia compared to vehicle treated rats as determined by one-way, repeated-measure ANOVA with post-hoc Dunnett's tests.

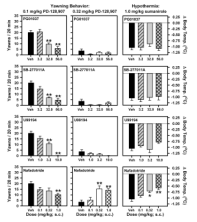

Figure 3.

Effects of the D3-preferring antagonists, PG01037 (0, 3.2, 32.0, and 56.0 mg/kg), SB-277011A (0, 3.2, 32.0, and 56.0 mg/kg), U99194 (0, 1.0, 3.2, and 10.0 mg/kg), and nafadotride (0, 0.1, 0.32, and 1.0 mg/kg) on yawning induced by 0.1 mg/kg PD-128,907 (left column), and 0.32 mg/kg PD-128,907 (center column), or hypothermia induced by 1.0 mg/kg sumanirole (right column). Antagonists were administered 30 min prior to agonist injections, and data are presented as mean (±SEM), n=8, number of yawns during a 20 minute observation period, and mean (±SEM), n=8, change in core body temperature as measured 30 min after, compared to 1 min before agonist injection. *p<0.05, **p<0.01. Significant difference from vehicle treated rats as determined by one-way, repeated-measure ANOVA with post-hoc Dunnett's tests.

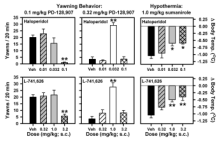

Figure 4.

Effects of the D2-preferring antagonists, haloperidol (0, 0.01, 0.032, and 0.1 mg/kg), and L-741,626 (0, 0.32, 1.0 and 3.2 mg/kg) on yawning induced by 0.1 mg/kg PD-128,907 (left column), and 0.32 mg/kg PD-128,907 (center column), or hypothermia induced by 1.0 mg/kg sumanirole (right column). Antagonists were administered 30 min prior to agonist injections, and data are presented as mean (±SEM), n=8, number of yawns during a 20 minute observation period, and mean (±SEM), n=8, change in core body temperature as measured 30 min after, compared to 1 min before agonist injection. *p<0.05, **p<0.01. Significant difference from vehicle treated rats as determined by one-way, repeated-measure ANOVA with post-hoc Dunnett's tests.

Table 1

in vivo D3 selectivity ratios determined from the minimal effective doses for D2/D3 agonist-induction, and antagonist-modulation of yawning and hypothermia.

	M.E.D. (mg/kg; s.c.)		
	***in vivo* D2**	***in vivo* D3**	***in vivo***
Compound	**Hypothermia**	**Yawning**	**D2/D3**
Agonists			
Pramipexole	0.32	0.01	32
PD-128,907	0.32	0.032	10
7-OH-DPAT	0.1	0.01	10
Quinpirole	0.1	0.01	10
Quinelorane	0.01	0.001	10
U91356A	0.32	0.1	3.2
Apomorphine	0.1	0.032	3.2
Sumanirole	0.32	(3.2)[a]	(0.1)[a]
ABT-724	*n.d.*[b]	*n.d.*[c]	*n.d.*[b,c]
PD-168,077	*n.d.*[b]	*n.d.*[c]	*n.d.*[b,c]
Antagonists			
PG01037	>56.0	32.0	*n.d.*[d]
SB-277011A	>56.0	32.0	*n.d.*[d]
U99194	>10.0	3.2	*n.d.*[d]
Nafadotride	0.32	1.0	0.32
Haloperidol	0.032	0.1	0.32
L-741,626	1.0	3.2	0.32

[a] $M.E.D._{D3}$ was not determined for sumanirole as the observed yawning was not sensitive to D3 antagonism.

[b] $M.E.D._{D3}$ could not be determined as compound failed to induce significant increases in yawning behavior.

[c] $M.E.D._{D2}$ could not be determined as compound failed to induce significant decreases in core body temperature.

[d] *in vivo* D3 selectivity ratio could not be determined as compound failed to significantly alter the induction of hypothermia by sumanirole at any dose tested.

Table 2

Effects of the D2 antagonist L-741,626 and the D3 antagonist PG01037 on D2/D3 agonist-induced yawning behavior[a]

	Vehicle	32.0 PG01037	1.0 L-741,626
Agonist	**Yawns (±SEM)**	**Yawns (±SEM)**	**Yawns (±SEM)**
Pramipexole – 0.1 mg/kg	24.6 (±2.3)	**6.6 (±3.6)	23.0 (±1.7)
0.32 mg/kg	8.0 (±2.0)	4.0 (±1.7)	**22.9 (±3.2)
PD-128,907 – 0.1 mg/kg	20.0 (±1.7)	**9.5 (±1.2)	21.6 (±3.6)
0.32 mg/kg	3.6 (±1.7)	2.1 (±0.7)	**27.6 (±3.1)
7-OH-DPAT – 0.032 mg/kg	22.5 (±4.9)	**6.5 (±2.3)	25.6 (±3.9)
0.1 mg/kg	4.9 (±0.4)	3.6 (±1.1)	**15.5 (±2.9)
Quinpirole – 0.032 mg/kg	18.3 (±1.1)	**4.9 (±1.1)	14.9 (±2.1)
0.1 mg/kg	5.3 (±1.0)	3.0 (±0.5)	**14.4 (±1.7)
Quinelorane – 0.0032 mg/kg	26.0 (±4.5)	**6.0 (±2.8)	21.5 (±1.7)
0.01 mg/kg	2.6 (±0.7)	2.8 (±0.9)	**17.4 (±3.0)
U91356A – 0.1 mg/kg	14.6 (±1.1)	**4.3 (±1.1)	16.8 (±1.4)
0.32 mg/kg	1.5 (±0.6)	1.1 (±0.1)	**9.6 (±1.9)
Apomorphine – 0.032 mg/kg	12.0 (±3.2)	**2.6 (±1.2)	13.4 (±2.4)
0.1 mg/kg	7.3 (±1.6)	4.1 (±1.1)	**17.5 (±2.1)
Sumanirole – 3.2 mg/kg	11.1 (±2.3)	8.6 (±1.3)	**19.4 (±0.9)

[a] Antagonists were given as 30 min pretreatments with the total number of yawns recorded during a 20 min period starting 10 min after agonist administration. Data are expressed as mean ±SEM, n=8 rats per group;

* $p<0.05$,

** $p<0.01$ with respect total yawns of antagonist treated rats compared to vehicle treated rats.

Table 3

Effects of the D2 antagonist L-741,626 and the D3 antagonist U99194 on D2/D3 agonist-induced hypothermia[a]

	Vehicle	1.0 L-741,626	3.2 U99194
Agonist	**Δ Temp. (±SEM)**	**Δ Temp. (±SEM)**	**Δ Temp. (±SEM)**
Pramipexole – 0.32 mg/kg	−1.50 (±0.11)	**−0.52 (±0.13)	−1.51 (±0.06)
PD-128,907 – 0.32 mg/kg	−1.30 (±0.12)	**−0.38 (±0.12)	−1.34 (±0.17)
7-OH-DPAT – 0.1 mg/kg	−1.15 (±0.23)	**−0.53 (±0.10)	−1.12 (±0.17)
Quinpirole – 0.1 mg/kg	−0.93 (±0.14)	*−0.23 (±0.15)	−0.84 (±0.22)
Quinelorane – 0.01 mg/kg	−0.73 (±0.07)	*−0.52 (±0.05)	−0.67 (±0.05)
U91356A – 0.32 mg/kg	−1.25 (±0.17)	**−0.58 (±0.12)	−1.29 (±0.18)
Apomorphine – 0.1 mg/kg	−0.74 (±0.13)	*−0.39 (±0.07)	−0.72 (±0.08)
Sumanirole – 1.0 mg/kg	−1.05 (±0.10)	*−0.50 (±0.07)	−1.09 (±0.13)
5-HT1A-preferring			
8-OH-DPAT – 1.0 mg/kg	−2.61 (±0.08)	−2.73 (±0.08)	−2.56 (±0.09)

[a] Antagonists were administered as 30 min pretreatments with Δ Temp. representing the change in core body temperature 30 min after, compared to 1 min prior agonist administration. Data are expressed as mean ±SEM, n=8 rats per group;

* $p<0.05$,

** $p<0.01$ with respect to Δ Temp of antagonist treated rats compared to vehicle treated rats.