Behavioral Pharmacology of Dopamine D_2 and D_3 Receptor Agonists and Antagonists in Rats.

by

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DEDICATION

This thesis is dedicated to my parents, Thomas and Shirley Collins, without whom none of this would have been possible. Your continual support and encouragement throughout all of my endeavors has meant more than you will ever know. Thank you.

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Abbreviations:

3-PPP: dl-3-[3-hydroxyphenyl]-N-n-propylpiperidine

5-HT: serotonin

6-OHDA: 6-hydroxydopamine

7-OH-DPAT: (±)-7-Hydroxy-2-dipropylaminotetralin

8-OH-DPAT: 8-Hydroxy-2-(di-n-propylamino)tetralin

A-437203: 2-{3-[4-(2-tert-butyl-6-trifluoromethyl-pyrimidin-4-yl)-piperazin-1-yl]-propyl-sulfanyl}-3*H*-pyrimidin-4-one-fumarate

ABT-724: 2-(4-pyridin-2-ylpiperazin-1-ylmethyl)-1*H*-benzimidazole

ACTH: adrenocorticotropin hormone

ALS: amyotrophic lateral sclerosis

apomorphine: (*R*)-(-)-5,6,6*a*,7-Tetrahydro-6-methyl-4*H*-dibenzo[*de*,*g*]quinoline- 10,11-diol hydrochloride

bromocriptine: (+)-2-Bromo-12'-hydroxy-2'-(1-methylethyl)-5'-(2-methylpropyl) ergotaman-3',6'-18-trione methanesulfonate

DA: dopamine

DOPA: 3,3-dihydroxyphenylalanine

ED_{50}: effective dose resulting in 50% maximal effect

GR218231: 2(*R*,*S*)-(di-*n*-propylamino)-6-(4-methoxyphenylsulfonylmethyl)-1,2,3,4-tetrahydronapthalene

haloperidol: 4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone hydrochloride

IC_{50}: concentration required to inhibit maximal response by 50%

L-741,626: 3-[[4-(4-Chlorophenyl)-4-hydroxypiperidin-l-yl]methyl-1*H*-indole

L-745,870: 3-(4-[4-Chlorophenyl] piperazin-1-yl)-methyl-1*H*-pyrrolo[2,3-b]pyridine trihydrochloride

MED: minimal effective dose

MK-801: (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine

NAcc: nucleus accumbens

nafadotride: N-[(1-Butyl-2-pyrrolidinyl)methyl]-4-cyano-1-methoxy-2-naphthalenecarboxamide

NMDA: *N*-methyl-D-aspartate

PCP: phencyclidine

PD-128,907: (*S*)-(+)-(4*aR*,10*bR*)-3,4,4*a*,10*b*-Tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano-[4,3-*b*]-1,4-oxazin-9-ol hydrochloride

PD-128,908: (*R*)-(–)-(4*aS*,10*bS*)-3,4,4*a*,10*b*-Tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano-[4,3-*b*]-1,4-oxazin-9-ol hydrochloride

PD-168,077: *N*-(Methyl-4-(2-cyanophenyl)piperazinyl-3-methylbenzamide maleate

PE: penile erection

PG01037: *N*-{4-[4-(2,3-Dichlorophenyl)-piperazin-1-yl]-*trans*-but-2-enyl}-4-pyridine-2-yl-benzamide hydrochloride

phMRI: pharmacologic magnetic resonance imaging

physostigmine: (3*aS*)-cis-1,2,3,3*a*,8,8*a*-Hexahydro-1,3*a*,8-trimethylpyrro lo [2,3-b]indol-5-ol methylcarbamate hemisulfate

PIP3EA: 2-[4-(2-methoxyphenyl)piperazin-1-ylmethyl]imidazo[1,2-*a*]pyridine

pramipexole: (*S*)-2-amino-4,5,6,7-tetrahydro-6-(propylamino)benzothiazole dihydrochloride

PVN: paraventricular nucleus

quinelorane: (5*aR*-trans)-5,5*a*,6,7,8,9,9*a*,10-Octahydro-6-propylpyrido [2,3-*g*]quinazolin-2-amine dihydrochloride

quinpirole: (4*aR*-trans)-4,4*a*,5,6,7,8,8*a*,9-Octahydro-5-propyl-1*H*-pyrazolo [3,4-*g*]quinoline hydrochloride

rCBV: regional cerebral blood volume

ropinirole: 4-(2-dipropylaminoethyl)-1,3-dihydroindol-2-one

S33084: (3*aR*,9*bS*)-*N*-[4-(8-cyano-1,3*a*,4,9*b*-tetrahydro-3*H*-benzopyro[3,4-*c*] pyrrole-2-yl)-butyl]-(4-phenyl) benzamide

SB-277011A: trans-N-[4-[2-(6-Cyano-1,2,3, 4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-4-quinolinecarboxamide

SCH 23390: (*R*)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride

SEM: standard error of the mean

siRNA: small interfering RNA

sumanirole: (*R*)-5,6-dihydro-5-(methylamino)-4*H*-imidazo[4,5,1-*ij*]quinolin-2(1*H*)- one (*Z*)-2-butenedioate

SSRI: serotonin selective reuptake inhibitor

TFMPP: *N*-[3-(Trifluoromethyl)phenyl]piperazine hydrochloride

TNPA: trihydroxy-*N*-n-propylnoraporphine

U91356A: (*R*)-5-(propylamino)-5,6-dihydro-4*H*-imidazo-[4,5,1-*ij*]quinolin-2(1*H*)-one hydrochlorinde

U99194: 2,3-Dihydro-5,6-dimethoxy-*N*, *N*-dipropyl-1*H*-inden-2-amine maleate

VTA: ventral tegmental area

ABSTRACT

Dopamine D_2-like receptors are involved in the regulation of a variety of behaviors, and have proven to be important pharmacologic targets for the treatment of diseases such as Parkinson's disease, schizophrenia, restless leg syndrome, and depression, however, the receptor(s) responsible for the therapeutic and behavioral effects have yet to be elucidated. Identification of behaviors specifically mediated by the D_2 and/or D_3 receptors would not only provide insight into the receptor(s) mediating these therapeutic and behavioral effects, but it would also aid in the evaluation of novel D_2-like agonists and antagonists. These studies were primarily aimed at the pharmacologic evaluation of the hypothesis that the induction of yawning by D_2-like agonists is mediated by a specific activation of the D_3 receptor, while the inhibition of yawning observed at higher doses is mediated by a concomitant activation of the D_2 receptor.

Convergent evidence from the effects of D_2-like agonists alone, and in combination with a series of D_2-like antagonists support this general hypothesis. All D_3-preferring agonists elicited dose-dependent yawning behavior resulting in a characteristic inverted U-shaped dose-response curve. These functions were differentially modulated by D_3- and D_2-preferring

antagonists, with D_3-preferring antagonists producing selective rightward shifts of the ascending limb, and D_2-preferring antagonists producing selective shifts of the descending limb. The selectivity of these effects was confirmed by a comparison of the relative potencies of D_2- and D_3-preferring agonists to induce yawning and hypothermia (a well validated D_2-mediated effect), as well as the relative potencies of D_2- and D_3-preferring antagonists to inhibit the induction of yawning and hypothermia by D_2-like agonists. Similar comparisons of the effects of D_2-like agonists and antagonists on the induction of yawning and penile erection not only provided further support for the differential roles of the D_3 and D_2 receptors in the regulation of yawning, but suggest that D_2-like agonist-induced yawning and penile erection are similarly mediated by the D_3 (induction) and D_2 (inhibition) receptors in rats. These studies not only provide strong pharmacologic evidence for a specific D_3-mediated behavior, but have also allowed for the identification of other D_3-mediated behaviors and determinations of *in vivo* D_2/D_3 selectivity.

CHAPTER I

General Introduction

Early Laboratory Work

While the catecholamine, 3-hydroxytyramine, was known to occur naturally in urine and heart (e.g., Holtz et al., 1947; Goodall, 1950), it was thought to be of little importance other than its role as a biosynthetic precursor of norepinephrine and epinephrine. It was not until 3,4-dihydroxyphenylalanine (DOPA) was shown to reverse the behavioral effects of reserpine in the rabbit (Carlsson et al., 1957), and the later discovery of large quantities of 3-hydroxytyramine in the rabbit brain (Carlsson et al., 1958), that 3-hydroxytyramine began to be thought of as the independent neurotransmitter we now refer to as dopamine. The capacity of DOPA to reverse the akinetic state induced by reserpine coupled with the finding that finding that the majority of the brain's dopamine was located in the striatum led Carlsson (1959) to hypothesize on a role of dopamine in the regulation of motor function. In fact, it was the similarities between the akinetic state induced by reserpine and that of Parkinson's patients, combined with the relatively low levels of dopamine observed in the caudate and putamen of post-mortem Parkinson's patients

(Ehringer and Hornykiewicz, 1960) that led to the initial trials which demonstrated the effectiveness of L-DOPA at reversing the symptoms of Parkinson's disease (Birkmayer and Hornykiewicz, 1961). However, it was not until L-DOPA was combined with an inhibitor of peripheral aromatic-L-amino-acid decarboxylase, the enzyme responsible for converting DOPA to dopamine, that a clinically effective, oral dosing procedure for the treatment of Parkinson's disease with L-DOPA was developed (Cotzias et al., 1967).

Shortly after the discovery of large quantities of dopamine in rabbit brain (Carlsson et al., 1958), the development of formaldehyde histofluorescence allowed for the visualization of catecholamine- (norepinephrine and dopamine) containing neurons in the central nervous systems of laboratory animals (Falck and Torp, 1962). It was through this method that Dahlstrom and Fuxe (1964) were able to produce the first detailed description of the catecholamine-containing neurons of the rat brain. This report identified twelve groups of catecholamine-containing cells which were distributed from the medulla oblongata to the hypothalamus, and designated A1 through A12 based on their anatomical orientation. Later studies identified five additional groups of catecholamine-containing cells resulting in the seventeen groups of cells now referred to as groups A1-A17. Of these seventeen groups of cells, groups A8-A17 represent dopaminergic cell groups, while groups A1-A7 represent noradrenergic cell groups. Subsequently, Ungerstedt (1971b) produced the first stereotaxic map detailing the dopaminergic pathways of the brain by combining the lesioning of distinct cell groups with formaldehyde histofluorescence.

Further advances in histochemical techniques, including the use of glyoxylic acid fluorescence (Lindvall and Bjorklund, 1974a) and more recently the use of immunohistochemical techniques for the identification of tyrosine hydroxylase (e.g., Hokfelt et al., 1976; Hokfelt et al., 1977), have allowed for the study of dopaminergic pathways with much greater resolution. While the nine major groups of dopaminergic neurons are still classified as A8-A17, these groups have been functionally divided into four main groups, the midbrain dopamine neurons comprised of groups A8-A10, the diencephalic dopamine neurons comprised of groups A11-A15, dopaminergic neurons in the olfactory bulb (A16), and the dopaminergic neurons located in the retina (A17). Figure 1.1 shows the distribution of the dopamine neuron cell groups in the rodent brain.

Dopaminergic Systems of the Central Nervous System

The mesencephalic, or midbrain dopaminergic system has been further sub-divided into three separate pathways, the nigrostriatal, mesolimbic, and mesocortical pathways, all of which originate from the A8-A10 cell groups. The nigrostriatal dopaminergic pathway originating primarily from the A9 group of the substantia nigra pars compacta, and to a lesser degree the A10 neurons of the ventral tegmental area (VTA) and A8 neurons of the retrorubal nucleus projects to a variety of structures within the dorsal striatum including the caudate, putamen, and globus pallidus, and is important in the regulation and coordination of locomotor activity (Ungerstedt, 1976). While the nigrostriatal pathway projects to the dorsal striatum, the mesolimbic dopaminergic pathway projects from the A8-A10 neurons to ventral striatum. However, unlike the nigrostriatal pathway, the majority of the neurons that make up the mesolimbic dopaminergic pathway originate from the A10 neurons of the VTA, with fewer neurons originating from the A8 and A9 groups, and project to the nucleus accumbens (NAcc), amygdala, and olfactory tubercle. In addition to its role in the regulation of affect, emotion, and locomotor activity, the mesolimbic dopaminergic pathway has also been shown to be involved in motivation and reinforcement, and is often referred to as the "reward pathway" of the brain. The third major dopaminergic pathway originating from the A8-A10 groups of neurons innervates a variety of cortical structures including the prefrontal cortex, pallidum, subthalamic nucleus, superior colliculus, and cerebral cortex, and is commonly referred to as the mesocortical pathway. Similar to the mesolimbic dopaminergic pathway, the majority of the neurons that comprise the mesocortical dopaminergic pathway originate from the A10 group of neurons within the VTA, with fewer neurons originating from the A9 group. While similarities in the origins of the mesolimbic and mesocortical dopaminergic pathways have led some to refer to these pathways as the mesolimbocortical dopaminergic pathway, neurons within the VTA are rarely double labeled in retrograde labeling studies (Swanson, 1982; Loughlin and Fallon, 1984) suggesting distinct populations of dopaminergic neurons project to the limbic and cortical structures described above. Furthermore, unlike the mesolimbic pathway, mesocortical dopaminergic neurons appear to be important for social behavior, working memory, attention, and executive function (e.g., Stam et al., 1989; Bubser and Schmidt, 1990; Sawaguchi and Goldman-Rakic, 1994; Robbins et al., 1998; Romanides et al., 1999; Floresco and Magyar, 2006).

The diencephalic dopaminergic system is composed of the A11-A15 groups of dopaminergic neurons located in the periventricular, hypothalamic, incertohypothalamic, and preoptic regions of the brain, and can be functionally subdivided into three main pathways, the diencephalospinal, incertohypothalamic, and tuberoinfundibular dopaminergic pathways. The diencephalospinal dopaminergic pathway originates in the A11 group of neurons located in the periventricular gray matter of the thalamus, and hypothalamus, and to a lesser degree the A13 group of neurons located in the zona incerta. The diencephalospinal pathway sends projections to the spinal cord and, to a lesser degree, the dorsal raphe nucleus and has been shown to be involved in dopamine-mediated nociception, and regulation of movement. The incertohypothalamic dopaminergic pathway originates from the A11, A13, and A14 groups of neurons and projects to the anterior and periventricular hypothalamus as well as the medial preoptic area, and has been shown to play a crucial role in the regulation of sexual behavior (Melis and Argiolas, 1995). The tuberoinfundibular dopaminergic pathway, originating in the A12 group of neurons and terminating in the median eminence of the pituitary, is involved in the regulation of reproductive processes, as well as the regulation and release of a variety of pituitary hormones, including prolactin.

Dopaminergic Receptors

While the existence of two types of dopamine receptor was first demonstrated in 1979 (Kebabian and Calne, 1979), it was not until the early 1990's that the D_3, D_4, and D_5 receptors were identified (Sokoloff et al., 1990; Sunahara et al., 1991; Van Tol et al., 1991). To date there are five known dopamine receptors, D_1-D_5, all of which are members of the G-protein coupled receptor super-family, but are subdivided into two families of dopamine receptors, the D_1-like and D_2-like families of dopamine receptors based on the G-proteins with which they couple, as well as their sequence homology. The D_1-like family (D_1 and D_5 receptors) are coupled to $G_{\alpha s}$ G-proteins with agonist activation resulting in increases in cAMP levels, while the D_2-like family, comprised of the D_2, D_3 and D_4 dopamine receptors, have been shown to couple $G_{\alpha i/o}$ G-proteins with agonist activation resulting in decreases in intracellular cAMP levels. Within the D_2-like family a high degree of sequence homology exists between the D_2 and D_3 receptors (52% overall and 75% in the transmembrane domains; Sokoloff et al., 1990), however, this high degree of sequence homology does not extend to the D_4 receptor which shares ~40% of the overall amino acid sequence, and only ~50% when the transmembrane domains are compared to those of both the D_2 and D_3 receptors (Van Tol et al., 1991). The differences in the sequence homology of transmembrane domains of the D_2, D_3, and D_4 receptors have influenced the availability of selective agonists and antagonists for the D_2, D_3, and D_4 receptors as these regions are thought to form the ligand binding domains. For example, although few agonists or antagonists exist with greater than 100-fold selectivity for either the D_2 or D_3 receptors (Heier et al., 1997; Stemp et al., 2000; Millan et al., 2002; Grundt et al., 2005), several agonist and antagonists with greater than 1000-fold selectivity for the D_4 receptor have been described (Glase et al., 1997; Patel et al., 1997; Cowart et al., 2004).

Although the levels of expression differ greatly (e.g., D2 ~2X greater than D3; Levesque et al., 1992), D_2-like receptors have been shown to have partially overlapping patterns of distribution. For example, while all three receptor subtypes are expressed to some degree within limbic regions of the brain, D_2 receptors possess a much more global pattern of distribution with relatively high levels of expression seen in almost all dopaminoceptive areas of the brain including both limbic (NAcc core, olfactory tubercle), and striatal (substantia nigra pars compacta, caudate-putamen, and globus pallidus) regions of the brain (Sokoloff et al., 1990; Bouthenet et al., 1991; Mengod et al., 1992; Gurevich and Joyce, 1999). Similar patterns of expression have been reported for D_4 receptors (Van Tol et al., 1991; Defagot et al., 1997). Unlike the D_2 and D_4 receptors, D_3 receptors display a much more restricted, limbic pattern of distribution in both the rat (Levesque et al., 1992; Defagot et al., 1997) and human brain (Lahti et al., 1995; Gurevich and Joyce, 1999) with high levels of expression observed in the NAcc shell, Islands of Calleja, and olfactory tubercle, while only moderate levels of expression are seen in striatal regions such as the substantia nigra pars compacta, ventral caudate-putamen, and globus pallidus.

Dopaminergic Diseases

Central dopaminergic systems are important for the regulation of a variety of processes including cognitive, (i.e., memory, attention, and problem solving), affective, and emotional states, motivation, and the coordination of movement. Due to the fact that dopaminergic systems are involved in the regulation of many different behaviors, dysregulations these systems have been implicated in a wide variety of disease states including movement disorders, such as Parkinson’s disease and restless leg syndrome, psychiatric disorders, such as schizophrenia and depression, as well as diseases of addiction, such as drug abuse and eating disorders. In fact, D_2-like antagonists have long-been known to possess antipsychotic activity (Anton-Stephens, 1954; Janssen et al., 1960; Madras et al., 1981), while D_2-like agonists are effective in the symptomatic treatment of both Parkinson’s disease (Calne et al., 1974; Kapoon et al., 1989; Molho et al., 1995) and restless leg syndrome (Lin et al., 1998; Bliwise et al., 2005). These effects, combined with the relatively high levels of expression within the basal ganglia has led many to hypothesize that the D_2 and D_3 receptors are of pharmacologic interest for the treatment of a variety of dopaminergic diseases (e.g., Joyce, 2001; Heidbreder et al., 2005; Newman et al., 2005). However, due to the relative lack of subtype selective agonists and antagonists, the receptor(s) mediating either the therapeutic or mechanistic effects are yet to be fully elucidated.

While the causes, and processes involved in the onset of Parkinson's disease are not well known, the neurodegeneration of the nigrostriatal dopaminergic pathway is thought to be responsible for the majority of the clinical symptoms of Parkinson's disease which include tremor, rigidity, akinesia, and postural instability. Since the initial discovery that L-DOPA was effective at the symptomatic treatment Parkinson's disease (Birkmayer and Hornykiewicz, 1961), L-DOPA has remained the primary therapeutic for the symptomatic treatment of the disease, despite the fact that L-DOPA does little to slow disease progression, and that long term treatment is commonly accompanied by the development of dyskinesias. While no animal model perfectly reproduces the progression and/or symptomology of Parkinson's disease, the unilateral lesioning of the substantia nigra with 6-hydroxydopamine (6-OHDA) has been a widely used model as it results in an almost complete degeneration of the nigrostriatal pathway and an asymmetry of movement and posture (Ungerstedt, 1968; Ungerstedt, 1971a), similar to that observed in Parkinson's patients. Furthermore, the motor effects are exaggerated following dopaminergic stimulation with direct- or indirect-agonists resulting in contralateral (opposite the lesioned side) and ipsilateral (toward the lesioned side) rotational behavior, respectively (Ungerstedt and Arbuthnott, 1970; Ungerstedt, 1971c). This model has proven to have good predictive validity in identifying dopaminergic agonists with therapeutic potential for the symptomatic treatment of Parkinson's disease. Interestingly, newer direct acting D_2-like agonists, such as pramipexole, are proving to be equally effective at alleviating the symptoms of Parkinson's disease while slowing the onset and/or reducing the severity of the dyskinesias that typically accompany the long-term use of L-DOPA (e.g., Montastruc et al., 1999; ParkinsonStudyGroup, 2000; Inzelberg et al., 2003; Jenner, 2003; Marras et al., 2004; Hauser et al., 2007). Moreover, several recent studies suggest that pramipexole has neurogenic effects and may actually slow, or even reverse, the neurodegeneration seen with Parkinson's disease (e.g., ParkinsonStudyGroup, 2000; Clarke and Guttman, 2002; Joyce et al., 2003; Van Kampen et al., 2004; Izumi et al., 2007; Joyce and Millan, 2007).

While Parkinson's disease is marked by a variety of severe motor symptoms, dopaminergic systems have also been implicated in other, less severe, movement disorders such as restless leg syndrome. Patients with restless leg syndrome report uncomfortable sensations in their legs when sitting still or lying down, a symptom that is typically exaggerated at night and results in the urge to move their legs (e.g., Hening et al., 2007; Karatas, 2007). Unlike Parkinson's disease which affects the nigrostriatal dopaminergic pathway, restless leg syndrome is thought to result from a dysfunction of the A11 neurons of the diencephalospinal pathway which modulate spinal excitability, and have been implicated in the sensory processing of the legs. While there are no validated animal models of restless leg syndrome, low doses of D_2-like agonists, namely pramipexole and ropinirole, are effective at the symptomatic treatment of restless leg syndrome in humans (Lin et al., 1998; Bliwise et al., 2005).

In addition to movement disorders such as Parkinson's and restless leg syndrome, dysregulation of dopaminergic systems has also hypothesized to be involved in the pathophysiology and/or symptomology of a variety of psychiatric conditions including schizophrenia, depression, and bipolar disorder. The observations that depletion of monoamines with reserpine produces negative affect, while psychostimulants, such as amphetamine, have mood enhancing effects (Freis, 1954; Ferguson, 1955; Cameron et al., 1965; Hurst et al., 1969) were the basis for the hypothesis that a reduced function of the mesolimbic dopaminergic pathway may, at least in part, underlie major depression (e.g., Schildkraut, 1965; Willner, 1997; Dunlop and Nemeroff, 2007). Interestingly, D_2-like agonists have also been shown to have antidepressant activity in animal models of depression (Basso et al., 2005; Brocco et al., 2006), as well as in depressed individuals (Goldberg et al., 1999; Corrigan et al., 2000; Ostow, 2002). While the receptor(s) mediating these antidepressant effects are currently unknown, studies in rats have suggested a role for the D_3 receptor. For example, increases in D_3 receptor expression within the NAcc have been observed following chronic treatment with a wide range of antidepressants including selective serotonin (5-HT) reuptake inhibitors, tricyclic antidepressants, monoamine oxidase inhibitors, as well as electroconvulsive shock (Lammers et al., 2000a; Lammers et al., 2000b). While these findings do not provide strong evidence that the D_3 mediates the antidepressant effects of these treatments, they do suggest that increased D_3 receptor activity may be involved in the alleviation of depression.

While D_2-like agonists are effective in the treatment of diseases involving low dopaminergic activity, D_2-like antagonists are known to be effective at treating diseases involving high levels of dopaminergic activity such as schizophrenia and addiction. Schizophrenia is a complex psychiatric disorder which is difficult to fully treat because it is marked by both positive symptoms, such as delusion and hallucination, and negative symptoms, such as apathy, anhedonia, disorganization and social isolation. While it is clear that other neurotransmitter systems (i.e., cholinergic, serotonergic, and glutamatergic systems) are also involved in schizophrenia, dysregulations of dopaminergic systems are thought to be involved in both the positive, and negative symptoms of schizophrenia (e.g., Dajas et al., 1983; Olney and

Farber, 1995; Yeomans, 1995; Meltzer, 1999). It has been hypothesized that the negative symptoms result from a hypoactivity of the mesocortical dopaminergic pathway, while the positive symptoms result from a hyperactivity of the mesolimbic dopaminergic pathway (e.g., Matthysse, 1973; Meltzer and Stahl, 1976; Snyder, 1976; Seeman, 1980; Davis et al., 1991). While the development of an animal model of schizophrenia has been difficult due to the complex nature of the symptoms, the two most commonly employed animal models are acute, high-dose, administration of the N-methyl-D-aspartate (NMDA) receptor antagonist, phencyclidine (PCP), and chronic, or sub-chronic, administration of amphetamine. Validation for these models is provided by the fact that both typical (e.g., haloperidol) and atypical (e.g., clozapine) antipsychotics are effective at reversing some of the behavioral effects observed in these animal models (Featherstone et al., 2007; Mouri et al., 2007), as well as the fact that psychostimulants (Janowsky and Davis, 1978) and NMDA antagonists (Lahti et al., 1995) intensify the symptoms of schizophrenia when administered to schizophrenic patients. While typical antipsychotics are effective at treating psychosis, their use is limited due to a number of side-effects such as tardive diskinesia, catalepsy, and hyperprolactinemia; effects that are thought to result from their antagonist activity at the D_2 receptor. It has recently been hypothesized that the D_3 receptor may provide a useful therapeutic target for the treatment of schizophrenia as the D_3 receptor displays a much more restricted limbic pattern of distribution, combined with the fact that most typical antipsychotics are roughly equipotent at D_2 and D_3 receptors. Thus, it is thought that antagonists selective for the D3 receptor may provide antipsychotic activity without the negative side-effects that generally accompany typical antipsychotics (e.g., Joyce, 2001).

It was the discovery that animals would repeat actions that were followed by electrical stimulation of specific regions of the brain (Olds and Milner, 1954) that led to the theory that there were specific “reward centers” in the brain. While the catecholamine theory of reward was first suggested in the early 1960’s (Poschel and Ninteman, 1963), it was significantly strengthened by the fact that the brain regions that maintained self-stimulation corresponded to the major catecholamine projections of the brain (Ungerstedt, 1971b; Lindvall and Bjorklund, 1974b), as well as the fact that inhibitors of catecholamine synthesis attenuated the self-administration of methamphetamine (Pickens et al., 1968; Davis and Smith, 1972). A specific role for dopamine, and more specifically the mesolimbic dopamine system was proposed based on the work of Roberts and colleagues (Roberts et al., 1977), who showed that 6-OHDA lesions of the NAcc resulted in a long-lasting, ~70% decrease in cocaine self-administration, while lesions of ventral noradrenergic neurons did not alter the rate of cocaine self-administration. Shortly thereafter, similar decreases in d-amphetamine self-administration were reported following 6-OHDA lesions of the NAcc (Lyness et al., 1979), further strengthening the notion that the mesolimbic dopaminergic pathway, and more specifically the

NAcc is important in the reinforcing effects of psychostimulants. Subsequent studies demonstrating that increases in NAcc dopamine levels are observed with a wide variety of drugs including amphetamine, cocaine, opiates, ethanol, barbiturates, and nicotine (Di Chiara and Imperato, 1986; Imperato et al., 1986; Di Chiara and Imperato, 1988) suggests that dopamine plays an important role in the effects of a variety of drugs of abuse.

The high levels of dopamine D_2 and D_3 receptor expression within the core and shell of the NAcc, respectively (Levesque et al., 1992; Gurevich and Joyce, 1999; Stanwood et al., 2000a), has led many to hypothesize that D_2-like receptors play important roles in the mediation of the reinforcing properties of drugs of abuse such as cocaine (e.g., Heidbreder et al., 2005; Le Foll et al., 2005; Newman et al., 2005). Support for this notion has been provided by the findings that D_2/D_3 receptor levels are inversely correlated with the positive reinforcing effects of psychostimulants. Briefly, human subjects with lower striatal D_2/D_3 receptor levels report more pleasant effects of methylphenidate compared with those with higher D_2/D_3 levels (Volkow et al., 1999; Volkow et al., 2002), while monkeys with lower striatal levels of D_2/D_3 receptors more readily self-administered cocaine compared with monkeys with higher striatal D_2/D_3 levels (Morgan et al., 2002). Further evidence for the involvement of D_2 and D_3 receptors in the reinforcing effects of drugs has been provided through the study of D_2/D_3 agonists and antagonists in a variety of operant procedures in animals. In drug discrimination experiments, D_2/D_3 agonists often generalize to cocaine-trained cues (Barrett and Appel, 1989; Terry et al., 1994; Barrett et al., 2001), suggesting that the D_2 and/or D_3 receptors may, at least in part, mediate the interoceptive effects of cocaine. In self-administration procedures, D_2/D_3 agonists maintain responding when substituted for cocaine in rats (Caine and Koob, 1993; Parsons et al., 1996; Collins and Woods, 2007), mice (Caine et al., 2002), and monkeys (Woolverton et al., 1984; Nader and Mach, 1996; Sinnott et al., 1999; Caine et al., 2002; Woolverton and Ranaldi, 2002). Moreover, D_2/D_3 agonists induce non-reinforced, drug-appropriate, responding when given as pretreatments in reinstatement procedures (Khroyan et al., 2000; De Vries et al., 2002; Koeltzow and Vezina, 2005; Edwards et al., 2007), while antagonists at D_2/D_3 receptors have been shown to inhibit the capacity of drug-paired cues (Gilbert et al., 2005; Gal and Gyertyan, 2006; Cervo et al., 2007), stress (Xi et al., 2004), as well as drug “primes” (Andreoli et al., 2003; Xi et al., 2006) to reinstate responding. Taken together, these findings suggest that the D_2 and/or D_3 receptors play important roles the mediation of the reinforcing and/or interoceptive effects of a variety of drugs of abuse, and that the D_2 and/or D_3 receptors may provide a useful pharmacological target for the treatment of addiction disorders.

Since the initial discoveries that dopaminergic agonists and antagonists were effective at the symptomatic treatment of diseases such as Parkinson’s disease and schizophrenia, respectively, there has been a longstanding and sustained interest in the potential of dopamine, and in particular D_2-like, agonists and antagonists in the treatment of a variety of disease states. The development of longer acting ligands with modest improvements in selectivity (i.e., pramipexole), or reduced efficacy (i.e., aripiprazole) have proven to be equally effective, or improved, therapeutics with reduced side-effect profiles. However, due to the relative lack of sub-type selective agonists and antagonists, as well as well validated animal models of specific receptor activity, the receptor(s) mediating either the therapeutic or mechanistic effects are yet to be fully elucidated.

Behavioral Effects of D_2-like Agonists and Antagonists

While a great deal is known about the behavioral effects of D_2-like agonists and antagonists, the characterization and separation of *in vivo* effects specifically mediated by either the D_2 or D_3 receptor has been complicated by the lack of agonists and antagonists highly selective for either receptor subtype, as well as a lack of *in vitro* functional assays that are generally predictive for the D_2 and D_3 receptors. *In vivo* characterization has been further complicated by the large degree of variability in reported *in vitro* binding affinities and selectivities for both agonists and antagonists at the D_2 and D_3 receptors resulting from differences in methodology and assay conditions (Levant, 1997); the extremes of which are shown in table 1.1. While D_2-like agonists have been shown to induce yawning, penile erection, stereotypy, and changes in locomotor activity, body temperature, as well as certain neuroendocrine responses in addition to other behavioral measures (Faunt and Crocker, 1987; Millan et al., 1995a; Depoortere et al., 1996; Smith et al., 1997; Boulay et al., 1999a; Boulay et al., 1999b), few of these effects have been fully characterized and validated through both pharmacologic and genetic means. Perhaps the strongest evidence in support of a subtype specific *in vivo* effect is the induction of hypothermia resulting from agonist activation of the D_2 receptor.

D_2-like agonists produce dose-dependent decreases in body temperature, an effect that is observed following administration of relatively high doses of D_2-like agonists with a wide range of selectivities for the D_2 or D_3 receptor (Yehuda and Wurtman, 1972; Calne et al., 1975; Faunt and Crocker, 1987; Millan et al., 1994; Collins et al., 2007). The hypothermic effects of D_2-like agonists was first linked specifically to the D_2 receptor by Boulay and colleagues who showed that while D_3 receptor-deficient mice displayed a normal hypothermic response to 7-OH-DPAT and PD-128,907, the effect was completely absent in D_2 receptor-deficient mice suggesting that the induction of hypothermia by D_2-like agonists results from their activation of the D_2 but not D_3 receptor (Boulay et al., 1999a; Boulay et al., 1999b). Pharmacologic validation of these findings was later provided as the D_2-preferring antagonist L-741,626 significantly, and dose-dependently, inhibit the induction of hypothermia by the D_2-like agonist trihydroxy-N-n-propylnoraporphine (TNPA), while the D_3-preferring antagonist A-437203 was without effect at any dose tested (Chaperon et al., 2003). Together, these studies provide convergent evidence that the hypothermic effects of D_2-like agonists are mediated by their agonist actions at D_2 receptors in both rats and mice, however, the receptor(s) mediating other behavioral effects of D_2-like agonists are less clear.

While the hypothermic responses to D_2-like agonists are similar in rats and mice, these species appear to be differentially sensitive to the locomotor effects of D_2-like agonists. In mice, D_2-like agonists inhibit locomotor activity at doses lower than those required to induce hypothermia (Boulay et al., 1999a; Boulay et al., 1999b), and continue to suppress activity over a wide range of doses resulting in a monophasic dose-response curve (Pugsley et al., 1995; Pritchard et al., 2003). Studies in D_2 and D_3 receptor-deficient mice suggest that the D_2-like agonist-induced inhibition of locomotor activity in mice is mediated by the D_2 receptor, as the effect is observed in D_3 receptor-deficient mice, but absent in D_2 receptor-deficient mice (Boulay et al., 1999a; Boulay et al., 1999b). However, D_3 receptor-deficient mice display elevated levels of spontaneous locomotor activity compared to their wild-type littermates suggesting that the D_3 receptor may also involved in the inhibition of locomotor activity in mice (Accili et al., 1996; Xu et al., 1997).

Similar hypotheses have been made regarding the receptor regulation of locomotor activity in rats. However, unlike in mice, D_2-like agonists have typically been shown to have biphasic effects on locomotor activity in rats, with the inhibition of locomotor activity occurring at low doses, and stimulation of locomotor activity observed at higher doses, effects that are often attributed to the D_3 and D_2 receptors, respectively (Ernst, 1967; Mogilnicka and Klimek, 1977; Protais et al., 1983; Collins et al., 1989; Waters et al., 1993; Svensson et al., 1994; Pugsley et al., 1995; Smith et al., 1997; Pritchard et al., 2003; Millan et al., 2004). This hypothesis is further supported by the fact that D_2- and D_3-preferring antagonists have been shown to decrease and increase spontaneous locomotor activity, respectively. However, the fact that D_2- and D_3-preferring antagonists affect spontaneous locomotor activity has complicated the interpretation of their effects on D_2-like agonist-induced locomotor activity.

In addition to their effects on body temperature and locomotor activity, D_2-like agonists have also been shown to dose-dependently induce a variety of stereotyped behaviors in rats and mice including sniffing, gnawing, object chewing, vacuous chewing, and yawning (Ernst, 1967; Mogilnicka and Klimek, 1977; Protais et al., 1983; Collins et al., 1989; Smith et al., 1997). Similar to the hypothermic effects of D_2-like agonists, many of these behaviors are observed at doses higher than those required to inhibit locomotor activity suggesting that they may be mediated by D_2 receptor activation. While non-selective D_2-like antagonists are able to inhibit the induction of many of these behaviors, several of these effects, such as sniffing, have also been shown to be inhibited by D_1-like antagonists, an effect that is not observed with D_2-like agonist induced hypothermia, but is suggestive of a permissive role for the D_1 receptor in their mediation (Walters et al., 1987; Double and Crocker, 1990). However, unlike D_2-like agonist-induced sniffing and vacuous chewing, which are generally observed at doses that correspond to the stimulation of locomotor activity and induction of hypothermia, the induction of yawning behavior by D_2-like agonists is observed over a range of lower doses that generally correspond to their inhibitory effects on locomotor activity (Protais et al., 1983; Stahle and Ungerstedt, 1990; Stahle, 1992; Ferrari et al., 1993; Brus et al., 1995; Ferrari and Giuliani, 1995; Bristow et al., 1996; Smith et al., 1997).

Although yawning was first identified as a cholinergic response (Urba-Holmgren et al., 1977), the ability of dopaminergic agonists to induce dose-dependent increases in yawning behavior over low doses and subsequently inhibit the induction of yawning at higher doses in rats has been a long-studied phenomenon (e.g., Mogilnicka and Klimek, 1977; Holmgren and Urba-Holmgren, 1980; Yamada and Furukawa, 1980). Early hypotheses regarding the inverted U-shaped dose-response curves for yawning behavior attributed the increases in yawning behavior to agonist activity at pre-synaptic D_2 receptors, and the subsequent inhibition of yawning to agonist activity at post-synaptic D_2 receptors, or the concomitant activation of D_1 receptors (Yamada and Furukawa, 1980; Urba-Holmgren et al., 1982; Yamada et al., 1990). While this hypothesis supposes that the induction of yawning by dopamine agonists results from the increased cholinergic activity secondary to the activation of dopaminergic autoreceptors, there is considerable evidence to argue against the autoreceptor hypothesis of yawning. In a series of experiments, Stahl and Ungerstedt, demonstrated that the induction of yawning was not correlated with changes in synaptic dopamine levels, but rather occurred with a shorter latency, suggesting that yawning is mediated by postsynaptic receptor activation. This notion was also supported by the fact that pharmacologic manipulations that increased or decreased synaptic dopamine levels did not alter the capacity of D_2-like agonists to induce yawning (Stahle and Ungerstedt, 1987; Stahle and Ungerstedt, 1989b; Stahle and Ungerstedt, 1989a; Stahle and Ungerstedt, 1990; Stahle, 1992). Subsequent hypotheses proposed that the biphasic nature of yawning was mediated by multiple post-synaptic D_2 receptors with differing sensitivities (Stahle, 1992), however it was not until a role for the D_3 receptor in the mediation of D2-like agonist-induced hypolocomotion was proposed (Waters et al., 1993; Svensson et al., 1994) that the D_3 receptor was thought to be involved in the mediation of D_2-like agonist-induced yawning behavior (Levant, 1997). Around this time, it was reported that newly developed D_2-like agonists with relatively high degrees of selectivity for the D_3 receptor, such as PD-128,907 and 7-OH-DPAT induced yawning in a manner similar to that observed with non-selective dopamine agonists, such as apomorphine (Levesque et al., 1992; Pugsley et al., 1995; Khroyan et al., 1997). However, the specific dopamine receptor(s) involved in the regulation of dopaminergic agonist-induced yawning behavior could not be determined due to a lack of antagonists highly selective for either the D_2 or D_3 receptor sub-types.

Although the mechanisms involved in the regulation of dopamine agonist-induced yawning behavior received considerable attention during the mid-80's to early 90's, the specific mechanism remains unknown. Early studies demonstrated that dopaminergic agonists induced yawning as a result of the activation of dopamine receptors within the central nervous system as the induction of yawning was blocked by the centrally active D_2-like antagonist, sulpiride, but not the peripheral D_2 receptor antagonist, domperidone (Stahle and Ungerstedt, 1984). While several studies reported that microinjections of apomorphine into the striatum or septum induced yawning in rats (Dourish et al., 1985; Yamada et al., 1986), these studies typically administered 5 to 40X greater doses than those required to induce yawning following injection into the paraventricular nucleus (PVN) (Melis et al., 1987), an area of the brain that had previously been shown to mediate the induction of yawning by oxytocin (Melis et al., 1986). Subsequently, Melis and colleagues have confirmed that dopamine and oxytocin induce yawning through their actions in the PVN, however, they are only two of a growing number of neurotransmitters and neurohormones including acetylcholine, 5-HT, NMDA, nitric oxide, opioid peptides, and adrenocorticotropin (ACTH), that have been shown to be involved in the complex regulation of yawning behavior (Ferrari et al., 1963;

Urba-Holmgren et al., 1977; Roeling et al., 1991; Melis et al., 1992; Melis and Argiolas, 1993; Stancampiano et al., 1994).

Interestingly, while a variety of neurotransmitter and neurohormones affect yawning, antagonist studies have demonstrated a hierarchical order with regard to their involvement in the neuronal circuitry of yawning behavior. For example, yawning induced by dopaminergic agonists is blocked by D_2-like antagonists (e.g., Mogilnicka and Klimek, 1977; Yamada and Furukawa, 1980), oxytocin antagonists (Melis et al., 1989), nitric oxide inhibitors (Melis et al., 1994), and cholinergic antagonists (e.g., Holmgren and Urba-Holmgren, 1980; Yamada and Furukawa, 1980), but not serotonergic antagonists (Stancampiano et al., 1994). Conversely, cholinergic yawning is blocked by cholinergic antagonists (e.g., Urba-Holmgren et al., 1977), but not D_2-like, oxytocin, or serotonergic antagonists (Yamada and Furukawa, 1980; Yamada and Furukawa, 1981; Fujikawa et al., 1996b), while oxytocin-induced yawning is blocked by oxytocin and cholinergic antagonists, but not dopaminergic antagonists (Argiolas et al., 1986). Together with the results of microinjection and microdialysis studies, these studies suggest that dopaminergic-, glutamatergic-, and oxytocinergic-induced yawning results from an increased activation of oxytocinergic neurons originating in the PVN. These neurons innervate a variety of structures including the CA1 region of the hippocampus and medulla oblongata, and are thought to result in increases in cholinergic transmision (e.g., Argiolas and Gessa, 1991; Argiolas and Melis, 1998). While the precise cholinergic neurons mediateding the induction of yawning are not known, activation of the M_1 receptor (Fujikawa et al., 1996b) is thought to play an important role in the induction of yawning by dopaminergic, serotonergic, oxytocinergic, and serotonergic mechanisms (e.g., Yamada and Furukawa, 1980; Argiolas et al., 1986; Protais et al., 1995).

Similar to their capacity to induce yawning, D_2-like agonists also induce penile erection (PE) over a range of low doses in a variety of species including mice, rats, monkeys, and humans (Benassi-Benelli et al., 1979; Gisolfi et al., 1980; Lal et al., 1989; Rampin et al., 2003). Interestingly, the pro-erectile effects of D_2-like agonists are typically observed over a range of doses that also induce yawning and inhibition of locomotor activity, with the subsequent inhibition of PE observed at higher doses (Mogilnicka and Klimek, 1977; Melis et al., 1987; Ferrari and Giuliani, 1995). As with yawning, the pro-erectile effects of D_2-like agonists are thought to be centrally mediated as they are inhibited by relatively non-selective, centrally active, D_2-like antagonists such as haloperidol, sulpiride, and clozapine, but not the peripheral D_2-like antagonist domperidone (Benassi-Benelli et al., 1979; Gower et al., 1984; Doherty and Wisler, 1994; Hsieh et al., 2004). Moreover, a significant body of literature supports a common role for the paraventricular nucleus (PVN) in the induction of PE and yawning by both physiologic and pharmacologic means (e.g., Argiolas and Melis, 1998; Melis and Argiolas, 1999; Melis and Argiolas, 2003); however, the specific receptor(s) mediating the pro-erectile effects of

D_2-like agonists are yet to be elucidated. Recently, dose-dependent increases in PE have been reported following systemic and intra-PVN administration of a variety of D_4-selective agonists (Brioni et al., 2004; Hsieh et al., 2004; Melis et al., 2005; Enguehard-Gueiffier et al., 2006; Melis et al., 2006), suggesting the D_4 receptor may mediate the induction of PE by D_2-like agonists. This notion is further supported by the finding that PE induced by D_4-selective agonists, such as PD-168,077 and PIP3EA, are blocked by the D_4-selective antagonist, L745,870 (Melis et al., 2005; Enguehard-Gueiffier et al., 2006; Melis et al., 2006). However, D_4-selective agonists generally induce fewer erections compared to less selective D_2-like agonists such as apomorphine, and L-745,870 has been shown to be ineffective at altering the induction of PE by apomorphine (Melis et al., 2006), suggesting that other receptor(s) are also involved in the mediation of D_2-like agonist-induced PE.

Characterization of the receptor(s) involved in the mediation of elicited effects of non-selective drugs has provided valuable information with regard to the receptors mediating the behavioral, therapeutic, and adverse effects of drugs with diverse mechanisms of action. However, to date there is no well validated behavioral measure for assessing the action of agonists or antagonists at the dopamine D_3 receptor. Therefore, the primary goal of this thesis is to identify a behavior that is specifically mediated by the D_3 receptor in rats. Identification of such a behavior will allow for a more accurate interpretation of the effects of D_2-like agonists and antagonists and aid in the design and development of novel agonists and antagonists selective for the D_2 and/or D_3 receptors.

Specific Aims

Specific Aim 1: The aim of the first set of studies was to investigate the receptor regulation of dopaminergic yawning behavior by characterizing a series of D_2-like agonists with varying degrees of selectivity for the D_3 compared to D_2 receptor with respect to their capacity to dose-dependently induce yawning in rats. Likewise, a series of dopaminergic antagonists, including D_1-like, and D_2-, D_3-, and D_4-preferring antagonists, were assessed for their capacity to dose-dependently alter the dose-response curve for yawning induced by the prototypical D_3-preferring antagonist, PD-128,907. Additionally, the dopaminergic selectivity of the effects of D_3-preferring antagonists on yawning behavior was determined by comparing D_3-preferring, serotonergic, and cholinergic antagonists with respect to their capacity to alter the induction of yawning by the indirect-cholinergic agonist, physostigmine, the 5-HT_2 receptor agonist, TFMPP, and the D_3-preferring agonist, PD-128,907.

Specific Aim 2: The second set of studies were aimed at characterizing D_2-, D_3-, and D_4-preferring agonists with respect to their relative potencies to induced yawning, a putative D_3-mediated behavior, and hypothermia, a putative D_2-mediated effect, in rats. The capacity of antagonists selective for the D_2 and D_3 receptors to alter the induction of yawning and hypothermia by a series of D_2-like agonist was also assessed to characterize the involvement of the D_2 and D_3 receptors in the induction of yawning and hypothermia. Likewise, a series of non-selective D_2/D_3, and D_2- and D_3-preferring antagonists were also characterized with respect to their relative potencies to inhibit the induction of yawning by the D_3-preferring agonist, PD-128,907, and the induction of hypothermia by the D_2-preferring agonist, sumanirole. *In vivo* D_3 selectivity ratios for D_2-like agonists were determined using the induction of yawning and hypothermia as *in vivo* D_3 and D_2 potency measures, respectively. Similar determinations of *in vivo* selectivity were made for D_2-like antagonists using the inhibition of yawning and hypothermia as *in vivo* potency measures for the D_3 and D_2 receptors, respectively.

Specific Aim 3: The aim of the third set of studies was to characterize the receptor regulation of the pro-erectile effects of D_2-like agonists. To this end, a series of D_2-, D_3-, and D_4-preferring agonists were compared with respect to their capacity to dose-dependently induce yawning and penile erection in rats. Antagonist selective for the D_2 (L-741,626), D_3 (PG01037), and D_4 (L-745,870) receptors were then assessed for their capacity to selectively alter the dose-response curves for apomorphine- and pramipexole-induced yawning and penile erection. Finally, a series of D_2-like antagonists with a wide range of selectivities for the D_2, D_3 and D_4 receptors were assessed for their capacity to dose-dependently alter the induction of yawning and/or penile erection induced by the maximally effective dose of pramipexole.

Figure 1.1. Distribution of dopamine neuron cell groups in the rodent brain shown in a sagital view. The mesencephalic dopamine neuron cell groups (A8-A10) send projections to the striatum and cortex and are subdivided into the nigrostriatal, mesolimbic, and mesocortical dopaminergic pathways. The diencephalic dopamine neuron cell groups (A11-A15), and are subdivided into the diencephalospinal, incertohypothalamic, and tuberoinfundibular dopaminergic pathways. The A16 group of dopamine neurons are located in the olfactory bulb.

Figure 1.1. Distribution of dopamine neuron cell groups in the rodent brain

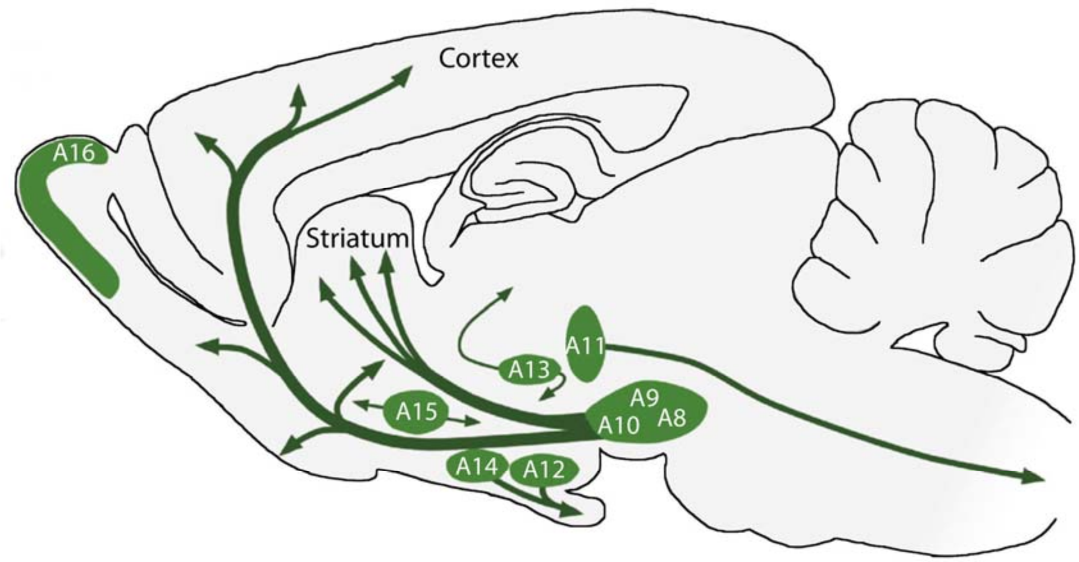


Table 1.1. Range of reported *in vitro* binding affinities at D_2 and D_3 receptors and D_3 selectivity ratios for D_2-like agonists and antagonists

	K_i D2 (nM)	**K_i D3 (nM)**	**D2/D3**
Agonists			
pramipexole	**3.9[a] - 955[b]**	**0.5[a] - 10.5[b]**	**2.1[c] - 488[d]**
PD-128,907	**42[e] - 389[f]**	**1.3[g] - 18[h]**	**6.1[h] - 340[g]**
7-OH-DPAT	**2.6[i] - 223[j]**	**0.34[g] - 7.1[j]**	**5.2[k] - 302[g]**
quinpirole	**1.8[a] - 1902[l]**	**0.86[f] - 410[m]**	**0.11[m] - 133[i]**
quinelorane	**5.7[h] - 708[b]**	**3.4[h] - 6.1[n]**	**1.7[h] - 131[b]**
U91356A	**1.6[o]- 875[p]**	**36[o]- 63.8[p]**	**0.044[o]- 13.7[p]**
apomorphine	**2.3[f] - 168[i]**	**2.2[f] - 73[f]**	**0.33[e] - 5.4[i]**
sumanirole	**9.0[q] - 53.8[r]**	**1940[s] - 2333[q]**	**0.0046[s] - 0.0038[q]**
ABT-724	**>10,000[t,r]**	**>10,000[t,r]**	**N.A.**
PD-168,077	**2810[u]**	**3740[u]**	**N.A.**
Antagonists			
PG01037	**93.3[v]**	**0.7[v]**	**133[v]**
SB-277011A	**1050[w] - 2820[w]**	**10.7[w] - 11.2[w]**	**94[w] - 263[w]**
U99194	**992[x] - 2281[g]**	**31[x] -160[g]**	**14.3[g] - 32[x]**
nafadotride	**3.0[y] - 7.0[h]**	**0.31[y] - 3.0[h]**	**2.3[h] - 9.7[y]**
haloperidol	**0.12[z] - 17[k]**	**0.2[aa] - 1020[k]**	**0.0043[z] - 3[aa]**
L-741,626	**2.4[bb] - 12[cc]**	**64[m] - 120[cc]**	**0.1[cc] - 0.024[bb]**

[a](Mierau et al., 1995); [b](Millan et al., 2002); [c](Seeman et al., 2005); [d](Gerlach et al., 2003); [e](Pugsley et al., 1995); [f](Sautel et al., 1995a); [g](Audinot et al., 1998); [h](Flietstra and Levant, 1998); [i](Burris et al., 1995); [j](MacKenzie et al., 1994); [k](Levant and DeSouza, 1993); [l](Kula et al., 1994); [m](Patel et al., 2003); [n](Millan et al., 1995b); [o](Piercey et al., 1996); [p](Kreiss et al., 1995); [q](Heier et al., 1997); [r](Brioni et al., 2004); [s](McCall et al., 2005); [t](Cowart et al., 2004); [u](Glase et al., 1997); [v](Grundt et al., 2005); [w](Reavill et al., 2000); [x](Haadsma-Svensson et al., 2001); [y](Sautel et al., 1995b); [z](Leopoldo et al., 2002); [aa](Burstein et al., 2005); [bb](Kulagowski et al., 1996); [cc](Bristow et al., 1998). Binding affinities represent the extremes of reported K_i values for agonist and antagonists for the D_2 and D_3 receptor. D_2/D_3 selectivity ratios represent the range of reported ratios as determined from binding studies in which affinities for both the D_2 and D_3 receptor were reported in the same publication.

CHAPTER II

Dopamine Agonist-Induced Yawning in Rats: A Dopamine D_3 Receptor Mediated Behavior.

Introduction

Dopamine D_3 receptors have received considerable interest since originally cloned (Sokoloff et al., 1990). The D_3 receptor shares significant sequence homology with the dopamine D_2 receptor, but displays a much more restricted, limbic pattern of distribution compared to that of the D_2 receptor in the rat (Levesque et al., 1992) and human brain (Gurevich and Joyce, 1999). Based in large part on this restricted distribution and high sequence homology, it has been hypothesized that the D_3 receptor may be of interest as a pharmacological target for antipsychotics and antiparkinsonian therapeutics (for review see Joyce, 2001). Additionally, the D_3 receptor is thought to play a role in reinforcement pathways, as the D_3 receptor is expressed in high levels within the mesolimbic dopaminergic system, and more specifically, the nucleus accumbens shell (Sokoloff et al., 1990; Stanwood et al., 2000a).

However, progress in defining a role for the D_3 receptor has been slowed by the inability to identify behavioral effects that can be linked exclusively to a D_3 mechanism (Levant, 1997). This is, at least in part, due to the lack of pharmacologically selective compounds acting at either the D_3 or D_2 receptors, as well as the fact that potentially selective agonists have failed to elicit obvious, direct behavioral changes. While D_2/D_3 agonists and antagonists have been shown to produce changes in body temperature, locomotor activity, and other behavioral measures (Millan et al., 1995a; Pugsley et al., 1995; Varty and Higgins, 1998), a role for the D_3 receptor in the regulation of these effects has typically not been confirmed by studies using D_3 receptor-deficient mice (Boulay et al., 1999a; Boulay et al., 1999b; Xu et al., 1999). Recently, increases in locomotor activity by MK-801 (Leriche et al., 2003) and blockade of the convulsant effects of dopamine uptake inhibitors (Witkin et al., 2004) have been proposed as *in vivo* models of D_3 receptor activation. However, systematic replication of those findings or confirmation by other means has not been reported. The studies reported herein provide evidence supporting the contention that yawning induced by D_2/D_3 agonists is mediated specifically through D_3 receptor activation.

The ability of dopaminergic agonists to elicit biphasic yawning resulting in an inverted U-shaped dose-response curve in rats has been a long-studied phenomenon (e.g., Mogilnicka and Klimek, 1977; Holmgren and Urba-Holmgren, 1980; Yamada and Furukawa, 1980). An early hypothesis regarding the biphasic regulation of apomorphine-induced yawning behavior attributed the induction of yawning behavior to a D_2 agonist activity, while the inhibition seen at higher doses was thought to be due to a competing D_1 agonist activation (Yamada and Furukawa, 1980; Urba-Holmgren et al., 1982). The cloning of the dopamine D_3 receptor and the development of agonists such as PD-128,907 (Pugsley et al., 1995) and 7-OH-DPAT (Levesque et al., 1992; Pugsley et al., 1995) as well as antagonists including U99194 (Cannon et al., 1982; Haadsma-Svensson et al., 2001), SB-277011A (Reavill et al., 2000; Stemp et al., 2000) and PG01037 (Grundt et al., 2005) with greater degrees of *in vitro* selectivity for the D_3 receptor have allowed greater insights into the regulation of dopaminergic agonist-induced yawning behavior to be made. Based on a series of studies examining the unconditioned behavioral effects of 7-OH-DPAT (Daly and Waddington, 1993; Ferrari and Giuliani, 1995; Kurashima et al., 1995), as well as binding studies (Levant et al., 1995), Levant (1997) hypothesized in an extensive review that, D_2/D_3 agonist-induced yawning may be a D_3 agonist-mediated effect, while the inhibition seen at higher doses was a result of concomitant D_2 agonist activation.

This hypothesis was evaluated in the present studies using a host of pharmacological tools. The abilities of a series of compounds with varying *in vitro* selectivities for the D_3 relative to D_2 receptors to elicit yawning were examined. A series of antagonists, again defined by binding *in vitro* selectivity for the D_3 and D_2 receptors, were evaluated with respect to their modification of dose-response relationships for D_2/D_3 agonists, with the majority of the studies using PD-128,907 as a prototype D_3 agonist.

Finally, in addition to dopaminergic mechanisms, yawning can be induced by cholinergic (Urba-Holmgren et al., 1977; Yamada and Furukawa, 1980) or serotonergic (Stancampiano et al., 1994) compounds. While the exact mechanisms and neural pathways involved in the regulation of yawning behavior have not been fully elucidated, there is a large set of data that suggests that dopaminergic, serotonergic, and cholinergic induction of yawning occur via distinct mechanisms. In addition both dopaminergic and serotonergic pathways are thought to eventually feed onto cholinergic neurons, thus allowing for differential regulation of dopaminergic and serotonergic yawning, with a cholinergic component common in all three pathways (for review see Argiolas and Melis, 1998). Therefore, some D3 antagonists that reduced PD-128,907-induced yawning were also assessed for their capacity to alter non-dopaminergic-induced yawning.

The convergent evidence from the agonist and antagonist studies support the hypothesis that dopamine agonist-induced yawning is mediated specifically through activation of D_3 receptors. Therefore, yawning in rats may provide a critical model for establishing the *in vivo* activities of putative D_3 selective ligands, a first step toward defining their role in normal and pathological physiological states.

Methods

Subjects: Male Sprague-Dawley rats (250-400 g) were obtained from Harlan (Indianapolis, IN) and housed three to a cage for the duration of the study. Rats had free access to standard Purina rodent chow and water, and were maintained in a temperature 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 Declaration of Helsinki and 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.

Behavioral Observations:

Yawning: 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 (19 in. x 9 in. x 8 in. clear "shoebox" rodent cage with standard cob bedding), and allowed to habituate to the chamber for a period of 30 minutes. Antagonist or vehicle was administered as a 30 minute pretreatment prior to the injection of agonist or vehicle. Behavioral observations began 10 minutes after all injections, and the total number of yawns was recorded for a period of 20 minutes thereafter. Dose-response curves were first generated for all agonists with a vehicle pretreatment, with antagonists substituted for vehicle pretreatments in subsequent sets of experiments. Each rat was tested multiple times, with separate groups of rats used to establish dose-response curves for each agonist, or antagonist X agonist combination. At least 48 hours was allowed between experimental sessions to allow for a drug washout period. Food and water were unavailable during individual test sessions, and all experiments were conducted between the hours of 12:00 PM and 6:00 PM.

Dopamine D_2/D_3 agonist-induced yawning: A series of dopaminergic agonists with varying degrees of *in vitro* selectivity for the D_3 and D_2 receptors were used to assess the ability of D_2/D_3 agonists to induce yawning behavior in rats. The D_2/D_3 agonists used in this series of experiments included: 7-OH-DPAT (0.0032, 0.01, 0.032, and 0.1 mg/kg), apomorphine (0.001, 0.0032, 0.01, 0.032, 0.1, and 0.32 mg/kg), bromocriptine (0.32, 1.0, 3.2, and 10.0 mg/kg), PD-128,907 (0.0032, 0.01, 0.032, 0.1, and 0.32 mg/kg), PD-128,908 (0.01, 0.032, 0.1, 0.32, and 1.0 mg/kg), pramipexole (0.00032, 0.001, 0.0032, 0.01, 0.032, 0.1, 0.32, and 1.0 mg/kg), quinelorane (0.0001, 0.00032, 0.001, 0.0032, 0.01, and 0.032 mg/kg), and quinpirole (0.0032, 0.01, 0.032, 0.1, and 0.32 mg/kg). All agonists were investigated in separate groups of rats, with doses presented in a random order.

Effects of dopaminergic antagonists on D_2/D_3 agonist-induced yawning behavior: The effects of dopaminergic antagonists on D_2/D_3 agonist-induced yawning were examined, with each antagonist X agonist combination determined in separate groups of rats. Agonist and antagonist dose combinations were presented in a random order, with dose-response curves for vehicle X agonist treatments determined before and after antagonist X agonist combinations to insure there were no changes in agonist-induced yawning behavior.

D_2-selective antagonists: The effects of L-741,626 (0.32 and 1.0 mg/kg) on yawning elicited by PD-128,907 (0.01, 0.032, 0.1, and 0.32 mg/kg), or quinelorane (0.00032, 0.001, 0.0032, 0.01, and 0.032 mg/kg) were determined. Only a dose of 1.0 mg/kg L-741,626 was used in the quinelorane-induced yawning studies.

Non-selective dopamine receptor antagonism: Haloperidol was used to determine the effects of non-selective dopaminergic antagonist activity on PD-128,907- (0.032, 0.1, 0.32, and 1.0 mg/kg) and quinelorane- (0.001, 0.0032, 0.01, 0.032, and 0.1 mg/kg) induced yawning. Doses of 0.01 and 0.032 mg/kg haloperidol were used in PD-128,907 experiments, while only the lowest active dose of 0.032 mg/kg was used in quinelorane studies.

D_3-selective antagonists: The D_3-preferring antagonists; nafadotride (0.01, 0.1, and 0.32 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 (10.0, 32.0, and 56.0 mg/kg) were used to examine their effects on PD-128,907- (0.01, 0.032, 0.1, and 0.32

mg/kg) induced yawning. In rats treated with 0.32 mg/kg nafadotride the range of doses used for PD-128,907-induced yawning was extended to 1.0 mg/kg.

D_1/D_5 and D_4-selective antagonists: The D_1/D_5 antagonist, SCH 23390 (0.01 mg/kg), and the D_4 antagonist, L-745,870 (3.2 mg/kg), were used to address the possible involvement of these receptors in yawning elicited by PD-128,907 (0.01, 0.032, 0.1, and 0.32 mg/kg).

Effects of cholinergic and serotonergic agonists and antagonists on yawning: Yawning elicited by cholinergic and serotonergic mechanisms were established by administration of physostigmine (0.01, 0.32, 0.1, 0.32, and 1.0 mg/kg; i.p.), and TFMPP (0.32, 1.0, 3.2, and 10.0 mg/kg) respectively. Scopolamine (0.0001, 0.001, and 0.01 mg/kg) was used to examine the effects of muscarinic cholinergic antagonism on yawning elicited by physostigmine (0.1 mg/kg; i.p.), TFMPP (3.2 mg/kg), and PD-128,907 (0.1 mg/kg). Likewise, the ability of the 5-HT_2 receptor antagonist mianserin (0.0032, 0.032, and 0.32 mg/kg) to antagonize yawning induced by TFMPP (3.2 mg/kg), physostigmine (0.1 mg/kg; i.p.), and PD-128,907 (0.1 mg/kg) was determined.

D_3-selective antagonists on cholinergic and serotonergic yawning: The ability of nafadotride (0.01, 0.1, 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 (10.0, 32.0, and 56.0 mg/kg) to modulate yawning behavior induced by PD-128,907

(0.1 mg/kg), TFMPP (3.2 mg/kg) and physostigmine (0.1 mg/kg; i.p.) was determined in separate groups of rats.

Drugs: (±)-7-OH-DPAT [(±)-7-Hydroxy-2-dipropylaminotetralin HBr], (-)-apomorphine [(*R*)-(-)-5,6,6*a*,7-Tetrahydro-6-methyl-4*H*-dibenzo[*de*,*g*]quinoline-10,11-diol HCl], (+)-bromocriptine [(+)-2-Bromo-12'-hydroxy-2'-(1-methylethyl)-5'-(2-methylpropyl) ergotaman-3',6'-18-trione methanesulfonate], haloperidol [4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone HCl], mianserin [1,2,3,4,10,14b-Hexahydro-2-methyldibenzo[c,f]pyrazino[1,2-a]azepine HCl], PD-128,907 [(*S*)-(+)-(4a*R*,10b*R*)-3,4,4a,10b-Tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano-[4,3-b]-1,4-oxazin-9-ol HCl], PD-128,908 [(*R*)-(–)-(4aS,10bS)-3,4,4a,10b-Tetrahydro-4-propyl-2H,5H-[1]benzopyrano-[4,3-b]-1,4-oxazin-9-ol HCl], physostigmine [(3a*S*)-*cis*-1,2,3,3a,8,8a-Hexahydro-1,3a,8-trimethylpyrrolo[2,3-b]indol-5-ol methylcarbamate hemisulfate], quinelorane [(5aR-trans)-5,5a,6,7,8,9,9a,10-Octahydro-6-propylpyrido[2,3-g]quinazolin-2-amine dihydrochloride], (-)-quinpirole [*trans*-(–)-(4aR)-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline HCl], SCH 23390 [(*R*)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine HCl], scopolamine [(*a*,S)-*a*-(Hydroxymethyl)benzeneacetic acid (1*a*,2*b*,4*b,*5*a*,7*b*)-9-methyl-3-oxa-9-azatricyclo[3.3.1.02,4]non-7-yl ester hydrobromide], and TFMPP [N-[3-(Trifluoromethyl)phenyl]piperazine HCl] were obtained from Sigma Chemical Co (St. Louis, Mo). L-741,626 [3-[[4-(4-Chlorophenyl)-4-hydroxypiperidin-l-yl]methyl-1*H*-indole], L-745,870 [3-(4-[4-

Chlorophenyl] piperazin-1-yl)-methyl-1*H*-pyrrolo[2,3-b]pyridine trihydrochloride], nafadotride [N-[(1-Butyl-2-pyrrolidinyl)methyl]-4-cyano-1-methoxy-2-naphthalenecarboxamide], and U99194 [2,3-Dihydro-5,6-dimethoxy-*N*, *N*-dipropyl-1*H*-inden-2-amine maleate] were obtained from Tocris (Ellisville, MO). Pramipexole [N'-propyl-4,5,6,7-tetrahydrobenzothiazole-2,6-diamine] was generously provided by Dr. Edward F. Domino, MD (University of Michigan Medical School, Ann Arbor, MI), SB-277011A [trans-N-[4-[2-(6-Cyano-1,2,3, 4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-4-quinolinecarboxamide] by Dr. Deyi Zhang (Lily Research Labs, Indianapolis, IN), and PG01037 [*N*-{4-[4-(2,3-Dichlorophenyl)-piperazin-1-yl]-*trans*-but-2-enyl}-4-pyridine-2-yl-benzamide HCl] by Dr. Amy H Newman (Medicinal Chemistry Section-NIDA, Baltimore, MD). All drugs were dissolved in sterile water with the exception of haloperidol, which was dissolved in 5% ethanol, L-741,626, which was dissolved in 5% ethanol with 1M HCl, and SB-277011A, which was dissolved in 10% β-cyclodextrin. All drugs were administered sub-cutaneously (s.c.) in a volume of 1 ml/kg, with the exception of physostigmine, which was administered i.p. 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: All yawning studies were conducted with 8 rats per group, and results are expressed as mean number of yawns during the 20 minute observation period ± standard error of the mean (SEM). A one-way, repeated-measures ANOVA with post-hoc Dunnett's tests was used to determine if agonist-induced yawning was significantly greater compared to vehicle (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Significant differences in the maximal amount of yawning elicited were determined by one-way repeated-measures ANOVA with post-hoc Tukey's HSD tests. Significant effects of antagonist pretreatment on agonist-induced yawning was determined using an unbalanced, two-way ANOVA with post-hoc Bonferroni tests to determine significant differences among antagonist and vehicle treated groups (SPSS, SPSS Inc., Chicago, IL). One-way repeated-measures ANOVAs with post-hoc Dunnett's tests were also used to determine if D3-preferring, cholinergic, or serotonergic antagonists significantly inhibited yawning elicited by the maximal effective dose of D_2/D_3, cholinergic, or serotonergic agonists (GraphPad Prism).

Results

Dopamine D_2/D_3 agonists on yawning behavior: D_2/D_3 agonists generally elicited dose-dependent increases in yawning behavior, with a subsequent inhibition of yawning seen at higher doses resulting in a characteristic inverted U-shaped dose-response curve as shown in figure 2.1. PD-128,907 [$F(5,35)=19.86$; $p<0.0001$], quinelorane [$F(6,42)=29.68$; $p<0.0001$], pramipexole [$F(8,56)=14.50$; $p<0.0001$], 7-OH-DPAT [$F(4,28)=39.68$; $p<0.0001$], quinpirole [$F(5,35)=42.47$; $p<0.0001$], and apomorphine [$F(6,42)=3.81$; $p<0.01$] all elicited significant, dose-dependent increases in yawning behavior compared to vehicle, while yawning induced by bromocriptine [$F(4,28)=1.14$; $p>0.05$] failed to reach significance. PD-128,908, the inactive enantiomer of PD-128,907 (DeWald et al., 1990) did not elicit yawning at any dose tested [$F(4,28)=0.30$; $p>0.05$]. Significantly greater amounts of yawning compared to vehicle were observed for PD-128,907 (0.032 and 0.1 mg/kg; $p<0.01$), quinelorane (0.001 and 0.0032 mg/kg; $p<0.01$), pramipexole [(0.01, 0.032 and 0.1 mg/kg; $p<0.01$); (0.32 mg/kg; $p<0.05$)], 7-OH-DPAT (0.01, and 0.032 mg/kg; $p<0.01$), quinpirole (0.01, and 0.032 mg/kg; $p<0.01$), and apomorphine (0.032 mg/kg; $p<0.05$).

There were no significant differences [$F(4,28)=1.70$; $p>0.05$] in the amount of yawning elicited by the maximal effective doses of PD-128,907 (0.1 mg/kg; 20.0±1.7), quinelorane (0.0032 mg/kg; 29.3±3.1), pramipexole (0.1 mg/kg; 24.5±4.4), 7-OH-DPAT (0.032 mg/kg; 23.4±3.0), and quinpirole (0.032 mg/kg; 27.5±2.9); however, the maximal effective dose of apomorphine (0.032 mg/kg; 10.4±3.1) [$F(5,42)=4.67$; $p<0.01$] produced significantly lower levels of yawning compared to all other D_2/D_3 agonists that elicited significant amounts of yawning.

D_2 selective-antagonism of D_2/D_3 agonist-induced yawning: The effects of L-741,626, a D_2-preferring antagonist approximately 50-fold selective for D_2 compared to D_3 receptors *in vitro* (Kulagowski et al., 1996), at behaviorally active doses (Chaperon et al., 2003), on PD-128,907- and quinelorane-induced yawning and are shown in Figures 2.2A and 2.2B respectively. An analysis of variance determined that there was an overall significant effect of L-741,626 on PD-128,907-induced yawning, and that the effect was dependent on both the dose of L-741,626 and PD-128,907 administered [main antagonist-dose effect, $F(2,103)=8.29$, $p<0.001$; main agonist-dose effect, $F(4,103)=20.34$, $p<0.001$; antagonist-dose x agonist-dose interaction, $F(6,103)=7.52$, $p<0.001$]. Likewise, L-741,626 significantly modified quinelorane-induced yawning, an effect that was dependent on both the dose of L-741,626, as well as the dose of quinelorane [main antagonist-dose effect, $F(1,79)=11.91$, $p<0.001$; main agonist-dose effect, $F(4,79)=18.64$, $p<0.001$; antagonist-dose x agonist-dose interaction, $F(4,79)=11.81$, $p<0.001$]. L-741,626 significantly increased the amount of yawning elicited by high doses of both PD-128,907 (0.32 mg/kg; $p<0.001$) and quinelorane (0.01 mg/kg; $p<0.001$), while having no effect on yawning induced by lower doses of either PD-128,907 or quinelorane.

Non-selective dopaminergic antagonism of D_2/D_3 agonist-induced yawning: Haloperidol, a non-selective dopaminergic antagonist with high affinities for all DA receptor subtypes (Sokoloff et al., 1992; Kulagowski et al., 1996), was used at behaviorally active doses (e.g., Leriche et al., 2003) to examine the effects of dopaminergic antagonism on yawning induced by PD-128907 and quinelorane (figures 2.2C and 2.2D, respectively). Pretreatment with haloperidol modified PD-128,907-induced yawning in a manner that was dependent on the dose of agonist administered [main antagonist-dose effect, $F(2,79)=1.86$, $p>0.05$; main agonist-dose effect, $F(3,79)=12.52$, $p<0.001$; antagonist-dose x agonist-dose interaction, $F(4,79)=21.30$, $p<0.001$]. The effects of haloperidol on quinelorane-induced yawning were similar to those on PD-128,907-induced yawning, and were dependent on both the dose of haloperidol and the dose of quinelorane [main antagonist-dose effect, $F(1,71)=10.78$, $p<0.01$; main agonist-dose effect, $F(4,71)=13.50$, $p<0.001$; antagonist-dose x agonist-dose interaction, $F(3,71)=22.55$, $p<0.001$]. Unlike L-741,626, haloperidol produced differential effects on D_2/D_3 agonist-induced yawning. Pretreatment with 0.032 mg/kg haloperidol resulted in significant decreases in yawning elicited by low doses of PD-128,907 (0.032 mg/kg; $p<0.05$) and quinelorane (0.001 mg/kg; $p<0.01$), while producing significant increases in the amount of yawning elicited by high doses of PD-128,907 (0.32 mg/kg; $p<0.001$) and quinelorane (0.01 and 0.032 mg/kg; $p<0.001$ and $p=0.001$ respectively).

D_3-preferring antagonists on D_2/D_3 agonist-induced yawning: Nafadotride, U99194, SB-277011A, and PG01037 have been shown to preferentially bind the D_3 receptor over the D_2 receptor *in vitro*, with D_3 selectivities of approximately 3-, 30-, 100-, and 133-fold respectively (Sautel et al., 1995b; Audinot et al., 1998; Flietstra and Levant, 1998; Stemp et al., 2000; Grundt et al., 2005), and were used at behaviorally active doses (Waters et al., 1993; Vorel et al., 2002; Di Ciano et al., 2003; Leriche et al., 2003; Millan et al., 2004) to examine their effects on yawning behavior in rats.

The effects of nafadotride (0.01, 0.1, and 0.32 mg/kg) on PD-128,907-induced yawning are shown in figure 2.3A. An analysis of variance revealed that nafadotride altered PD-128,907 induced yawning in a manner that was dependent on the dose of agonist administered [main antagonist-dose effect, $F(3,135)=0.34$, $p>0.05$; main agonist-dose effect, $F(4,135)=20.48$, $p<0.001$; antagonist-dose x agonist-dose interaction, $F(9,135)=3.92$, $p<0.001$]. While slight reductions in yawning elicited by low doses of PD-128,907 were observed with doses of 0.1 and 0.32 mg/kg nafadotride, these effects were not significant at either dose. However, pretreatment with 0.32 mg/kg nafadotride did produce significant increases in yawning elicited by 0.32 mg/kg of PD-128,907 ($p<0.001$).

The effects of U99194 (1.0 mg/kg, 3.2 mg/kg, and 10.0 mg/kg) on PD-128,907-induced yawning are shown in figure 2.3B. U99194 modified PD-128,907-induced yawning in a manner that was dependent on both the dose of U99194 and dose of PD-128,907 [main antagonist-dose effect, $F(3,119)=40.08$, $p<0.001$; main agonist-dose effect, $F(3,119)=42.26$, $p<0.001$; antagonist-dose x agonist-dose interaction, $F(8,119)=4.69$, $p<0.001$]. At a dose of 3.2 mg/kg, U99194 decreased the amount of yawning elicited by low doses of PD-128,907 (0.032 and 0.1 mg/kg; $p<0.05$ for both) while there was no effect on yawning elicited by 0.32 mg/kg PD-128,907. At the highest dose of U99194 tested (10.0 mg/kg), PD-128,907-induced yawning was completely inhibited at all doses tested [(0.032 mg/kg; $p<0.001$); (0.1 mg/kg; $p<0.001$) and (0.32 mg/kg; $p>0.05$)].

The effects of SB-277011A (3.2, 32.0 and 56.0 mg/kg) on PD-128,907-induced yawning are shown in figure 2.3C, and they were dependent on both the dose of SB-277011A as well as the dose of PD-128,907 administered [main antagonist-dose effect, $F(3,119)=29.18$, $p<0.001$; main agonist-dose effect, $F(3,119)=37.29$, $p<0.001$; antagonist-dose x agonist-dose interaction, $F(8,119)=4.40$, $p<0.001$]. SB-277011, at a dose of 32.0 mg/kg, significantly inhibited PD-128,907-induced yawning at doses corresponding to the ascending limb of the dose-response curve [(0.01 mg/kg; $p<0.05$); (0.032 mg/kg; $p=0.001$); and (0.1 mg/kg; $p<0.001$)]. Likewise, 56.0 mg/kg SB-277011 further reduced PD-128,907 elicited yawning at both 0.032 ($p<0.001$) and 0.1 mg/kg ($p<0.001$). There were no effects of any dose of SB-277011A on yawning induced by a high dose of 0.32 mg/kg PD-128,907.

PG01037, a D_3-preferring antagonist with similar *in vitro* selectivity for the D3 receptor compared to SB-277011A, was administered at doses of 10.0, 32.0, and 56.0 mg/kg, and the effects on PD-128,907 elicited yawning are shown in figure 2.3D. Pretreatment with PG01037 altered PD-128,907-induced yawning in a manner that was dependent on both the dose of PG01037 and dose of PD-128,907 administered [main antagonist-dose effect, $F(3,119)=17.68$, $p<0.001$; main agonist-dose effect, $F(3,119)=33.10$, $p<0.001$; antagonist-dose x agonist-dose interaction, $F(8,119)=2.69$, $p<0.05$]. Similar to SB-277011A, PG01037, at a dose of 32.0 mg/kg, significantly reduced yawning elicited by low doses of PD-128,907 [(0.032mg/kg; $p<0.01$) and (0.1 mg/kg; $p<0.001$)]. Further decreases in yawning induced by low doses of PD-128,907 [(0.032mg/kg; $p<0.001$) and (0.1 mg/kg; $p<0.001$)] were observed with a dose of 56.0 mg/kg PG01037. There were no effects of any dose of PG01037 on yawning induced by a high dose of 0.32 mg/kg PD-128,907.

Other dopamine receptor antagonists: The D_1-like receptor selective antagonist SCH 23390 (Barnett et al., 1986) and the D_4 selective antagonist L-745,870 (Kulagowski et al., 1996) were used at behaviorally active doses (Patel et al., 1997; Chaperon et al., 2003) to assess the ability of D_1/D_5 and D_4 antagonism respectively, to modulate the dose response curve for D_2/D_3 agonist-induced yawning. SCH 23390, at a dose of 0.01 mg/kg did not produce any significant change in the amount of yawning elicited by any dose of PD-128,907 tested (0.01 – 0.32 mg/kg; data not shown). Likewise, at a dose of 3.2 mg/kg, the D_4-selective antagonist L-745,870 failed to alter PD-128,907-induced yawning at any dose tested (0.01 – 0.32 mg/kg; data not shown).

Cholinergic- and serotonergic- induced yawning: Both physostigmine [$F(4,28)=7.11$; $p<0.001$] and TFMPP [$F(4,28)=7.15$; $p<0.001$]

also elicited, inverted U-shaped, dose-dependent yawning behavior in rats, as shown in figure 2.4A; however, both the cholinergic and serotonergic agonists were significantly less effective compared to PD-128,907 [F(2,14)=9.50; p=<0.01]. Maximal amounts of yawning induced by physostigmine and TFMPP occurred at doses of 0.1 mg/kg i.p, and 3.2 mg/kg, respectively, and were the only doses to elicit significantly greater amounts of yawning compared to vehicle treated rats (p<0.01 for both).

The effects of the non-selective, muscarinic antagonist, scopolamine (0.0001, 0.001, and 0.01 mg/kg), on yawning elicited by physostigmine (0.1 mg/kg; i.p.), PD-128,907 (0.1 mg/kg), and TFMPP (3.2 mg/kg) are shown in figure 2.4B. Scopolamine produced significant, dose-dependent antagonism of physostigmine-induced yawning [F(3,21)=16.89; p<0.0001], with a dose of 0.01 mg/kg scopolamine significantly inhibiting physostigmine-induced yawning compared to vehicle treated rats (p<0.01). In addition, scopolamine dose-dependently, and significantly inhibited yawning elicited by both PD-128,907 [F(3,21)=17.25; p<0.0001], and TFMPP [F(3,21)=22.40; p<0.0001]. Significantly lower levels of PD-128,907-induced yawning were observed with doses of 0.001, and 0.01 mg/kg scopolamine (p<0.01 for both). Scopolamine significantly reduced TFMPP elicited yawning at all doses tested (p<0.01 for all).

The effects of the 5-HT_2 receptor subtype antagonist, mianserin (0.0032, 0.032, and 0.32 mg/kg), on yawning elicited by TFMPP (3.2 mg/kg), PD-128,907 (0.1 mg/kg), and physostigmine (0.1 mg/kg; i.p.) are shown in figure 2.4C. Mianserin produced a dose-dependent and significant inhibition of TFMPP-induced yawning [$F(3,21)=9.85$; $p<0.001$], with doses of 0.032 and 0.32 mg/kg mianserin significantly inhibiting TFMPP-induced yawning compared to vehicle treated rats ($p<0.01$ for both). Mianserin did not significantly effect yawning elicited by either PD-128,907 [$F(3,21)=0.84$; $p>0.05$] or physostigmine [$F(3,21)=0.26$; $p>0.05$], at any dose tested.

D_3-preferring antagonists on dopaminergic, cholinergic and serotonergic agonist induced yawning: Figure 2.5 shows the effects of the D_3-preferring antagonists; nafadotride, U99194, SB-277011A, and PG01037 on yawning elicited by PD-128,907 (0.1 mg/kg), physostigmine (0.1mg/kg; i.p.), and TFMPP (3.2 mg/kg). Nafadotride (figure 2.5A), dose-dependently and significantly inhibited yawning elicited by PD-128,907 [$F(3,21)=5.36$; $p<0.01$) with a dose of 1.0 mg/kg significantly reducing yawning compared to vehicle treated rats ($p<0.01$). There were no significant effects of nafadotride on either physostigmine- [$F(3,21)=0.32$; $p>0.05$] or TFMPP- [$F(3,21)=0.60$; $p>0.05$] induced yawning. As shown in figure 2.5B, U99194 dose dependently and significantly reduced the amount of yawning elicited by PD-128,907 [$F(3,21)=29.78$; $p<0.0001$], with doses of 3.2 and 10.0 mg/kg U99194 significantly inhibiting yawning compared to vehicle treated rats ($p<0.01$ for both). Unlike nafadotride, U99194 also significantly inhibited the amount of yawning elicited by physostigmine [$F(3,21)=11.91$; $p<0.0001$], and TFMPP [$F(3,21)=7.07$; $p<0.01$], with a dose of 10.0 mg/kg U99194 resulting in a significant reductions in the amount of yawning elicited by both physostigmine ($p<0.01$) and TFMPP ($p<0.01$). The effects of SB-277011A on PD-128,907-, physostigmine-, and TFMPP-induced yawning are shown in figure 2.5C. SB-277011A dose-dependently and significantly reduced the amount of yawning elicited by PD-128,907 [$F(3,21)=12.09$; $p<0.0001$], with doses of 32.0 and 56.0 mg/kg ($p<0.01$ for both) significantly inhibiting yawning compared to vehicle treated rats. No significant effects of SB-277011A were seen on yawning elicited by either physostigmine [$F(3,21)=0.68$; $p>0.05$] or TFMPP [$F(3,21)=2.20$; $p>0.05$]. Similarly, PG01037 significantly and dose-dependently inhibited yawning elicited by PD-128,907 [$F(3,21)=29.43$; $p<0.0001$], with doses of 32.0 and 56.0 mg/kg ($p<0.05$ for both) PG01037 significantly reducing yawning compared to vehicle treated rats (figure 2.5D). PG01037 did not significantly effect yawning elicited by either 0.1 mg/kg physostigmine [$F(3,21)=0.16$; $p>0.05$], or 3.2 mg/kg TFMPP [$F(3,21)=0.07$; $p>0.05$] at any dose tested.

Discussion

Evidence has been provided in the present paper to support the hypothesis that D_2/D_3 agonist-induced yawning behavior in rats is mediated by agonist activation of the dopamine D_3 receptor, while the inhibition of yawning is a result of a competing agonist activation of the dopamine D_2 receptor. In agreement with the majority of previous studies, all of the D_2/D_3 agonists tested with exception of bromocriptine and PD-128,908 (Figure 2.1C), the inactive enantiomer of PD-128,907, elicited significant, dose-dependent increases in yawning behavior with inhibition seen at higher doses, resulting in the characteristic inverted U-shaped dose response curve for yawning in rats. Evidence is also provided for the selective antagonism of the induction of yawning behavior by D_3-preferring antagonists, and the inhibition of yawning by D_2-preferring antagonists. In addition, the current studies demonstrate that inhibition of D_3 agonist-induced yawning by D_3-preferring antagonists is a result of their selective antagonist activity at the D_3 receptor, and not through antagonist effects at D_2, serotonergic, or muscarinic cholinergic receptors.

Yawning is a D_3-mediated behavior: Several lines of evidence have been provided in support of the hypothesis that yawning is a D_3 agonist-mediated behavior. In general, all D_3-preferring D_2/D_3 agonists induced significant amounts of yawning at low doses. While there were no significant differences in the effectiveness of the agonists with respect to induction of yawning behavior with the exception of apomorphine, there were differences in the potency of the D_2/D_3 agonists to induce yawning. The rank-order potency of the D_2/D_3 agonists to elicit yawning behavior was as follows; quinelorane, apomorphine, quinpirole, 7-OH-DPAT, pramipexole, and PD-128,907, while bromocriptine and PD-128,908 failed to elicit significant levels of yawning. The stereoselectivity of the yawning response with regard to PD-128,907 [and PD-128,908] is an important finding, as dopamine receptors are selective with respect to more rigid agonists (DeWald et al., 1990). Taken together with the findings of Stahle and Ungerstedt (1984), who showed that (+)-3-PPP, but not (-)-3-PPP, will elicit yawning, our current findings provide further evidence that D_2/D_3 agonists are inducing yawning via dopaminergic agonist mechanisms. Differences in yawning induced by bromocriptine may be a result of pharmacokinetic differences, as bromocriptine has been shown to induce significant levels of yawning in studies using a 60 minute observation period (Protais et al., 1983; Zarrindast and Jamshidzadeh, 1992).

Antagonists with a high degree of selectivity for the D_3 compared to the D_2 receptor selectively antagonized the induction of yawning behavior. Three of the four D_3-preferring antagonists (U99194, SB-277011A, and PG01037) tested in the current studies possess the ability to dose-dependently and selectively antagonize the induction of yawning by PD-128,907, while having no effect on the inhibition of yawning observed at higher doses. As shown in figures 2.3C and 2.3D, respectively, SB-277011A and PG01037, D3-preferring antagonists with similarly high degrees of *in vitro* D_3 selectivity (100- and 133-fold respectively) produced almost identical effects on PD-128,907-induced yawning; significant, dose-dependent, downward/rightward shifts of the ascending limb of the yawning dose-response curve were observed, while the descending limb of the dose-response curve for PD-128,907-induced yawning was not changed. Similar effects were seen with the moderately selective (30-fold) D_3-preferring antagonist U99194, however, unlike SB-277011A and PG01037, at relatively high-dose of 10.0 mg/kg, U99194 completely inhibited PD-128,907-induced yawning; however, it should be noted that at this dose U99194 effectively antagonized not only dopaminergic, but cholinergic and serotonergic yawning as well. Nafadotride, the least selective (3-fold) of the D3-preferring antagonists, was the only D_3 antagonist to produce a non-selective antagonism of yawning behavior; shifting both the ascending and descending limbs of the dose-response curve for PD-128,907-induced yawning at the highest dose tested. This effect was similar to that observed with haloperidol, a non-selective dopamine antagonist, and suggests that at a dose of 0.32 mg/kg, nafadotride is no longer selective for the D_3 receptor, but rather is active at both the D_3 and D_2 receptors. Taken together, these data provide strong support for the hypothesis that the induction of yawning by D_2/D_3 agonists is mediated by an agonist activation of the D_3 receptor.

Inhibition of yawning is a D_2-mediated effect: We have also provided evidence in support of the hypothesis that inhibition of D_2/D_3 agonist-induced yawning occurring at higher doses is mediated by an agonist activity at the D_2 receptor. As shown in figure 2.2A and 2.2B, the D_2-preferring antagonist L-741,626, at the first behaviorally active dose (1.0 mg/kg), selectively antagonized the inhibitory effects of high doses of PD-128,907 and quinelorane, resulting in a rightward shift in the descending limbs while having virtually no effect on the ascending limbs of the dose-response curves for both PD-128,907- and quinelorane-induced yawning. In addition L-741,626 produced a rightward shift in the maximal effective dose of PD-128,907 and quinelorane, resulting in an increased effectiveness for both agonists. These data not only suggest that L-741,626, at a dose of 1.0 mg/kg, is an effective D_2 antagonist *in vivo*, but that it is also devoid of D_3 antagonist activity.

Further support for the differential regulation of yawning behavior by the D_3 and D_2 receptors was provided by the effects of the non-selective DA antagonist haloperidol. As D_3- and D_2-preferring antagonists selectively antagonize the ascending and descending limbs of the dose-response curve for D_2/D_3 agonist-induced yawning respectively, it would be expected that antagonists with mixed D_2/D_3 actions, such as haloperidol, would shift both the ascending and descending limbs of yawning dose-response curves at their initial active doses. Indeed, at the first behaviorally active dose (0.032 mg/kg), haloperidol produced rightward shifts in both the ascending and descending limbs of the dose-response curves for both PD-128,907- and quinelorane-induced yawning (Figures 2.2C and 2.2D). This not only suggests that the effects of D_3- and D_2-preferring antagonists are a result of selective antagonist activity, but that non-selective D_2/D_3 antagonists produce effects distinct from those of other dopaminergic antagonists on D_3 agonist-induced yawning.

However, it should be noted that in addition to possessing high affinities for the D_3 and D_2 receptors, haloperidol also has significant affinities for the D_1, D_4, and D_5 receptors. It is, however, unlikely that activity at these receptors is influencing PD-128,907-induced yawning behavior as the D_1/D_5-selective antagonist, SCH 23390, and the D_4-selective antagonist, L-745,870, at behaviorally active doses (Patel et al., 1997; Chaperon et al., 2003) did not alter yawning elicited by either low (0.032-0.1 mg/kg) or high (0.32 mg/kg) doses of PD-128,907. This provides further evidence that D_2/D_3 agonist-induced yawning behavior is under the direct control of the D_3 (induction) and D_2 (inhibition) receptors, but not the D_1, D_4, or D_5 receptors. However, the possibility remains that other dopaminergic receptors may modulate D_3 agonist-induced yawning elicited by other D_2/D_3 agonists, as several of the agonists tested, such as apomorphine, quinelorane, and quinpirole, possess significant affinities for the D_1, D_4, and D_5 receptors (apomorphine), or D_4 receptor (quinelorane and quinpirole) in addition to the D_3 and D_2 receptors.

Dopaminergic, serotonergic, and cholinergic regulation of yawning: The findings of the current study confirm, and extend those of earlier studies (e.g., Yamada and Furukawa, 1980; Ushijima et al., 1984; Zarrindast and Poursoltan, 1989; Stancampiano et al., 1994), and demonstrate that while scopolamine will dose-dependently antagonize yawning induced by cholinergic, serotonergic, and dopaminergic agonists (figure 2.4B), serotonergic and dopaminergic antagonists are able to selectively antagonize yawning elicited by their respective agonists. More specifically, nafadotride, SB-277011A, and PG01037, D_3-preferring antagonists with a wide range (3-133 fold) of selectivities for the D3 receptor over the D_2 receptor *in vitro*, were able to selectively antagonize PD-128,907-induced yawning, while having no effect on yawning elicited by either physostigmine or TFMPP (Figure 2.5). This suggests that SB-277011A and PG01037 are not only selective for the D_3 over the D_2 receptor, but that they are also selective for the D_3 receptor over certain serotonergic and cholinergic receptors at doses up to 56.0 mg/kg. Similarly, while nafadotride demonstrated little or no preference for the D_3 compared to the D_2 receptor *in vivo*, no serotonergic or cholinergic antagonist activity was detected at doses up to 1.0 mg/kg. However, in contrast to the effects of the other D_3-preferring antagonists, U99194, at a dose of 10.0 mg/kg, significantly antagonized yawning elicited by PD-128,907, TFMPP and physostigmine, suggesting that at higher doses, it is no longer selective for dopaminergic receptors. While U99194 is unique in this regard within this group of D_3-preferring antagonists, clozapine, an antagonist with significant affinities for dopaminergic, serotonergic and cholinergic receptors has also been shown to antagonize both dopaminergic and cholinergic yawning (Dubuc et al., 1982), suggesting that antagonism of physostigmine-induced yawning may be a reliable measure of anti-cholinergic activity. Further evidence of an *in vivo* anti-muscarinic activity of U99194 has been demonstrated by Goudie and colleagues (2001) who showed in discrimination studies that U99194 generalized to a scopolamine cue, suggesting that U99194 may possess anti-cholinergic activity at higher doses. Although it has been suggested that U99194 functions as a D_3 selective antagonist *in vivo* at doses ranging from 13.0 to 40.0 mg/kg based on its inability to increase plasma prolactin, to induce catalepsy, and to inhibit the induction of hypothermia by PD-128,907 (Audinot et al., 1998), the results of the current study suggest that while U99194 may be selective for the D_3 compared to the D_2, a significant anti-cholinergic effect is apparent at 10.0 mg/kg. Thus the current studies support the hypothesis that dopaminergic, serotonergic and cholinergic agonists induce yawning via distinct mechanisms, and furthermore that yawning induced by D_2/D_3 agonists is a result of agonist activation of D_3 receptors, and not serotonergic or cholinergic receptors.

To summarize the results of the studies reported herein, evidence has been provided in support of the hypothesis that the induction of yawning by D_2/D_3 agonists is mediated through an agonist activity at the D_3 receptor, while the subsequent inhibition of yawning seen at higher doses is a result of an increasing D_2 agonist activity. Based on these findings several conclusions can be drawn: First, the ascending limb of the dose-response curves corresponds to doses that are selectively activating D_3 receptors over D_2 receptors, while the descending limb corresponds to those activating both the D_3 and D_2 receptors. Additionally, determinations of *in vivo* D_3 potency and effectiveness may be possible, based on the onset and maximal amount of yawning elicited. Furthermore, inhibition of yawning may provide useful information regarding *in vivo* D_2 potency, and lastly, the shape of the dose-response curves may allow for determinations of *in vivo* D_3 selectivity of D_3-preferring D_2/D_3 agonists to be made. The results of the current set of studies have demonstrated that D_3 selective antagonism will only shift the ascending limb of the yawning dose-response curve, that D_2 selective antagonism will only shift the descending limb of the yawning dose-response curve, while non-selective D_2/D_3 antagonism will shift both the ascending and descending limbs of the dose-response curve for D_2/D_3 agonist-induced yawning behavior in rats. In conclusion, as the current studies have provided evidence that the induction of yawning behavior by D_2/D_3 agonists is mediated by the D_3 receptor, yawning may be an important pharmacological effect that can be used in the characterization, classification, and discovery of *in vivo* D_3 agonist and antagonist actions. Thus, it may be possible to relate other behavioral effects of D_2/D_3 agonists and antagonists to their ability to modulate yawning. Whether the potency and selectivity measures of these compounds can be utilized across behavioral measures will need to be explored in the future.

Figure 2.1. Dose-dependent induction of yawning by dopamine D_3-preferring agonists A) PD-128,907 (0.0032 – 0.32 mg/kg), quinelorane (0.0001 – 0.032 mg/kg), and pramipexole (0.00032 – 1.0 mg/kg); B) PD-128,907 (0.0032 – 0.32 mg/kg), 7-OH-DPAT (0.0032 – 0.1 mg/kg), and quinpirole (0.0032 – 0.32 mg/kg); C) PD-128,907 (0.0032 – 0.32 mg/kg), bromocriptine (0.32 – 10.0 mg/kg), apomorphine (0.001 – 0.32 mg/kg), and PD-128,908 (0.01 – 1.0 mg/kg). Data are presented as mean (±SEM), n=8, number of yawns during a 20 minute observation period.

Figure 2.1. D_2-like agonist-induced yawning in rats

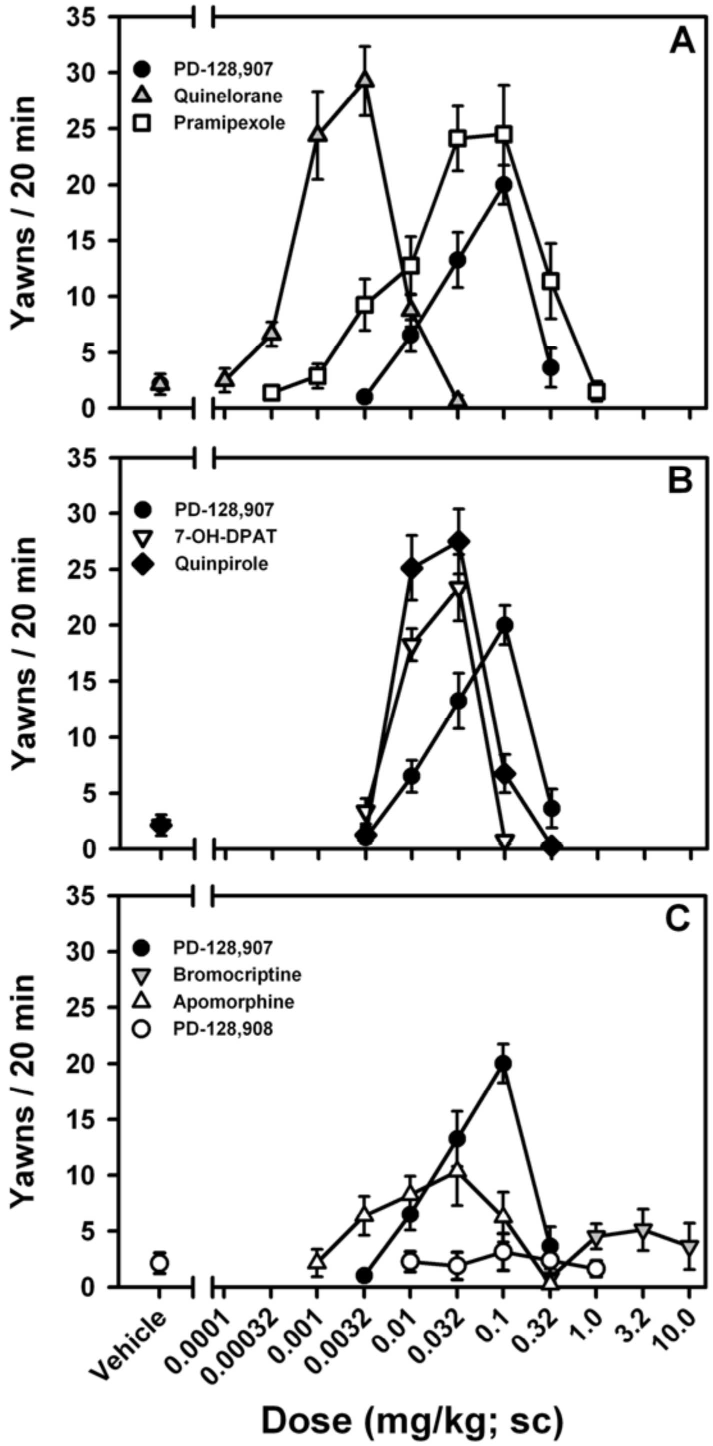


Figure 2.2. Effects of the D_2-selective antagonist L-741,626 (0.32 and 1.0 mg/kg) on A) PD-128,907 (0.0032 – 1.0 mg/kg) induced yawning, and B) quinelorane (0.0001 – 0.032 mg/kg) induced yawning. Effects of the non-selective dopamine receptor antagonist haloperidol (0.01 and 0.032 mg/kg) on C) PD-128,907 (0.0032 – 1.0 mg/kg) induced yawning, and D) quinelorane (0.0001 – 0.1 mg/kg) induced yawning. Data are presented as mean (±SEM), n=8, number of yawns during a 20 minute observation period. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; Significant difference from vehicle-treated animals was determined by unbalanced, two-way ANOVA with post-hoc Bonferroni tests.

Figure 2.2. Effects of D_2-preferring and non-selective D_2/D_3 antagonists on D_2-like agonist-induced yawning in rats

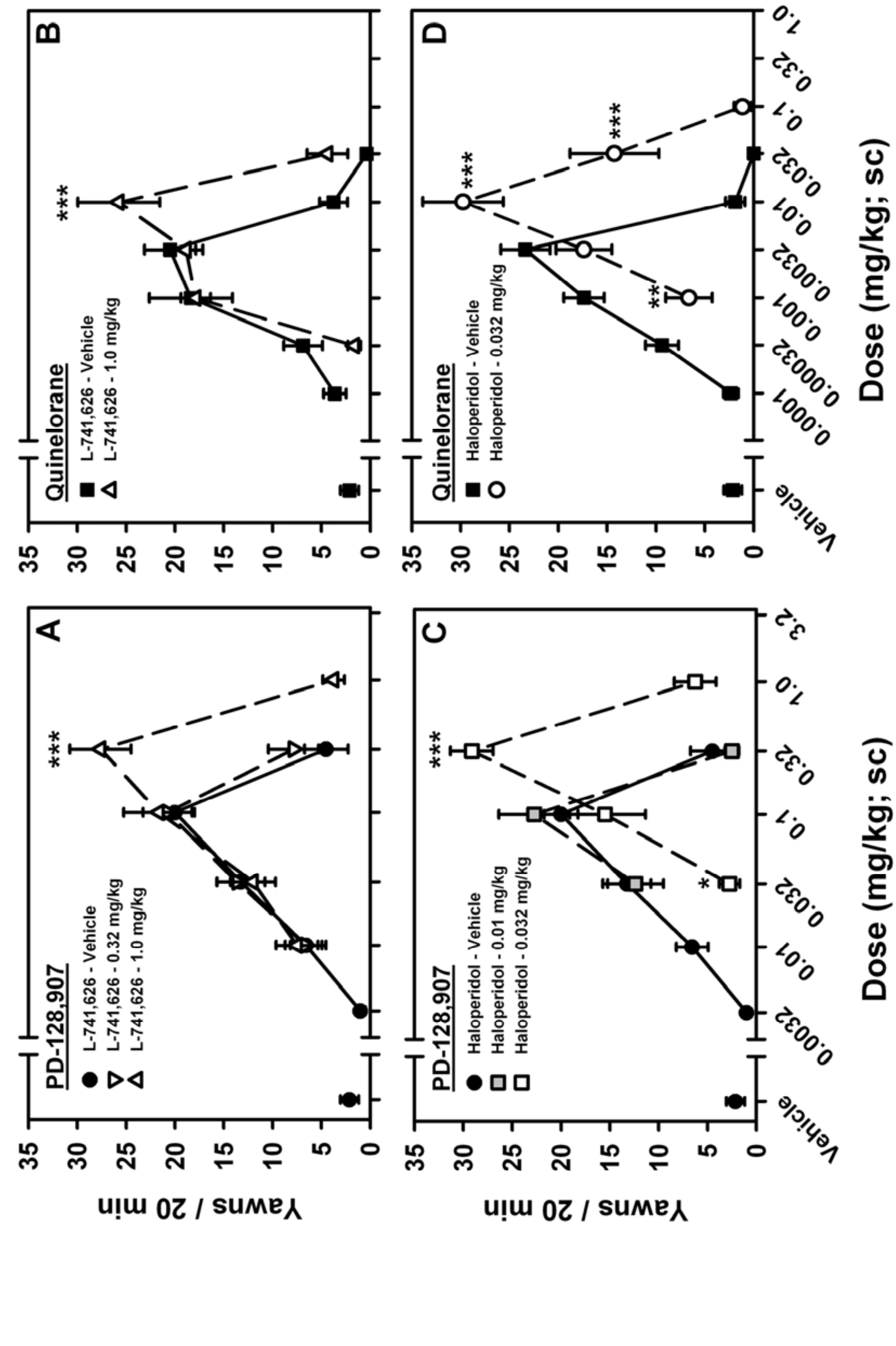


Figure 2.3. Effects of D_3-preferring antagonists on PD-128,907 (0.0032 – 0.32 mg/kg) induced yawning in rats. A) Nafadotride at doses of 0, 0.001, 0.1, and 0.32 mg/kg; B) U99194 at doses of 0, 1.0, 3.2, and 10.0 mg/kg; C) SB-277011A at doses of 0, 3.2, 32.0, and 56.0 mg/kg; and D) PG01037 at doses of 0, 10.0, 32.0, and 56.0 mg/kg. Data are presented as mean (±SEM), n=8, number of yawns during a 20 minute observation period. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; Significant difference from vehicle-treated animals was determined by unbalanced, two-way ANOVA with post-hoc Bonferroni tests.

Figure 2.3. Effects of D_3-preferring antagonists on D_2-like agonist-induced yawning in rats

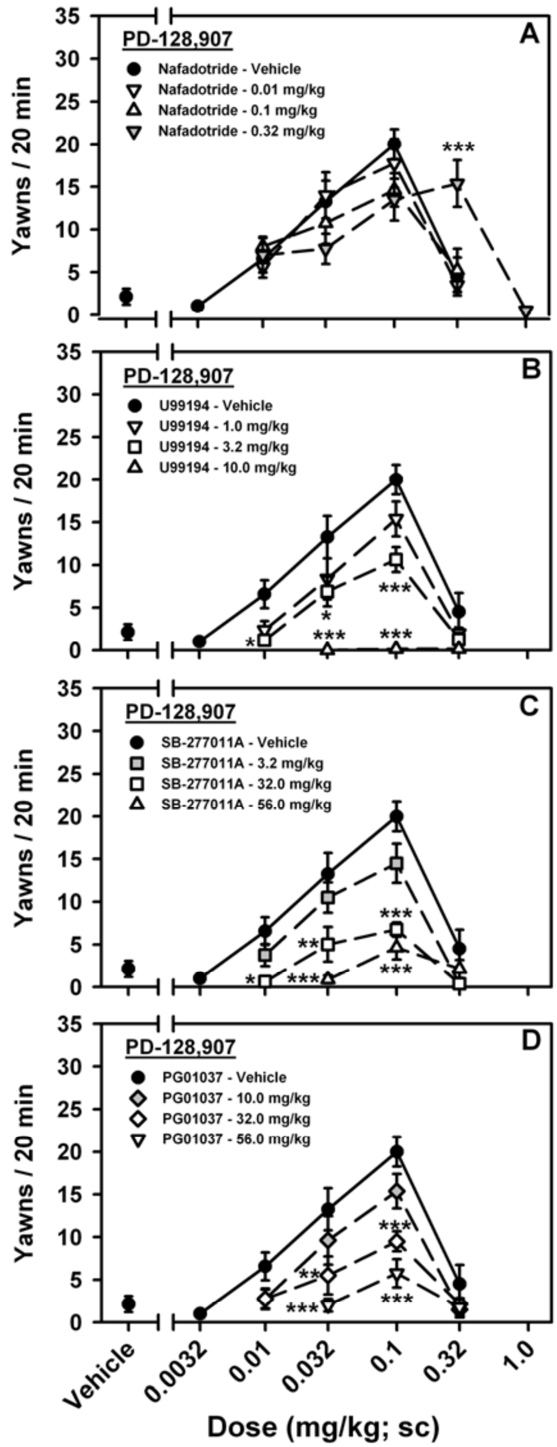


Figure 2.4. A) Dose-response curves for PD-128,907 (0.0032 – 0.32 mg/kg), physostigmine (0.01 – 1.0 mg/kg; i.p.), and TFMPP (0.32 – 10.0 mg/kg) induced yawning in rats. B) Effects of scopolamine (0, 0.0001, 0.001, and 0.01 mg/kg) on yawning induced by PD-128,907 (0.1 mg/kg), physostigmine (0.1 mg/kg; i.p.) and TFMPP (3.2 mg/kg). C) Effects of mianserin (0, 0.0032, 0.032, and 0.32 mg/kg) on yawning induced by PD-128,907 (0.1 mg/kg), physostigmine (0.1 mg/kg; i.p.) and TFMPP (3.2 mg/kg). Data are presented as mean (±SEM), n=8, number of yawns during a 20 minute observation period. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; Significant difference from vehicle-treated rats was determined by one-way repeated-measures ANOVAs with post-hoc Dunnett's tests.

Figure 2.4. Effects of cholinergic and serotonergic antagonists on yawning in rats

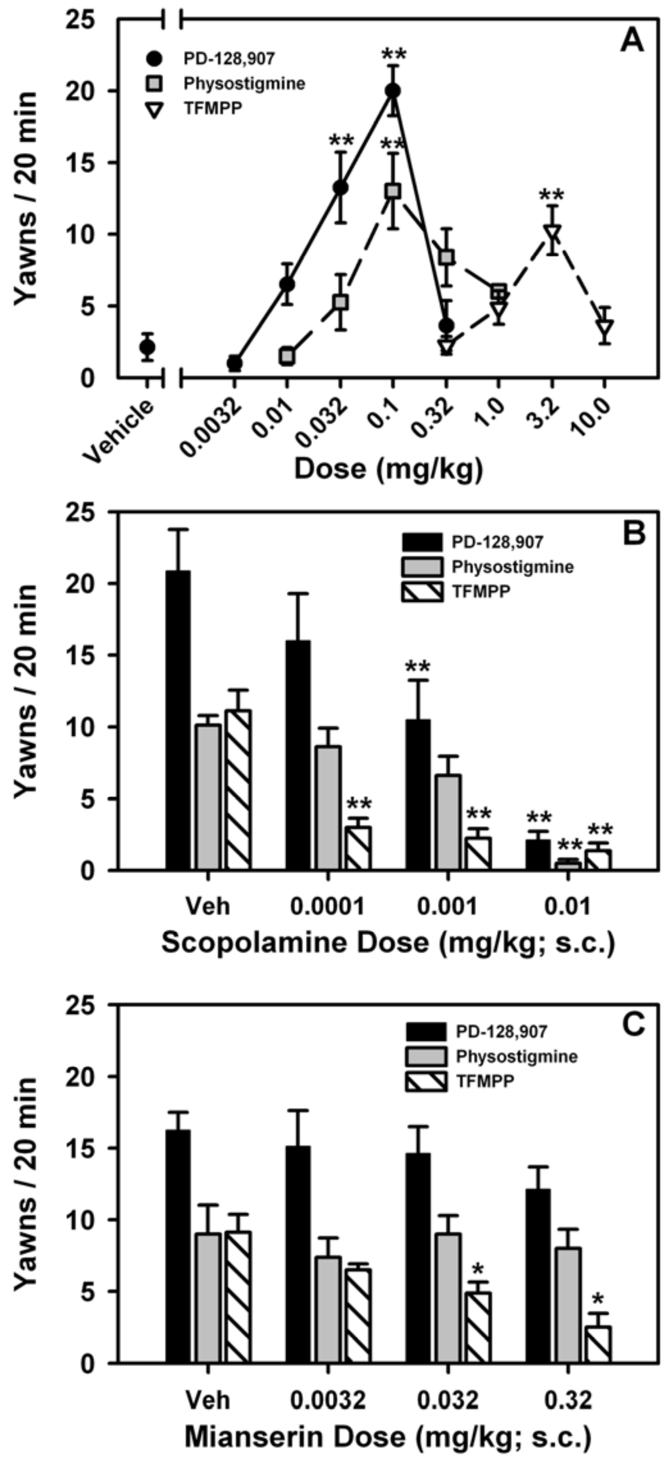
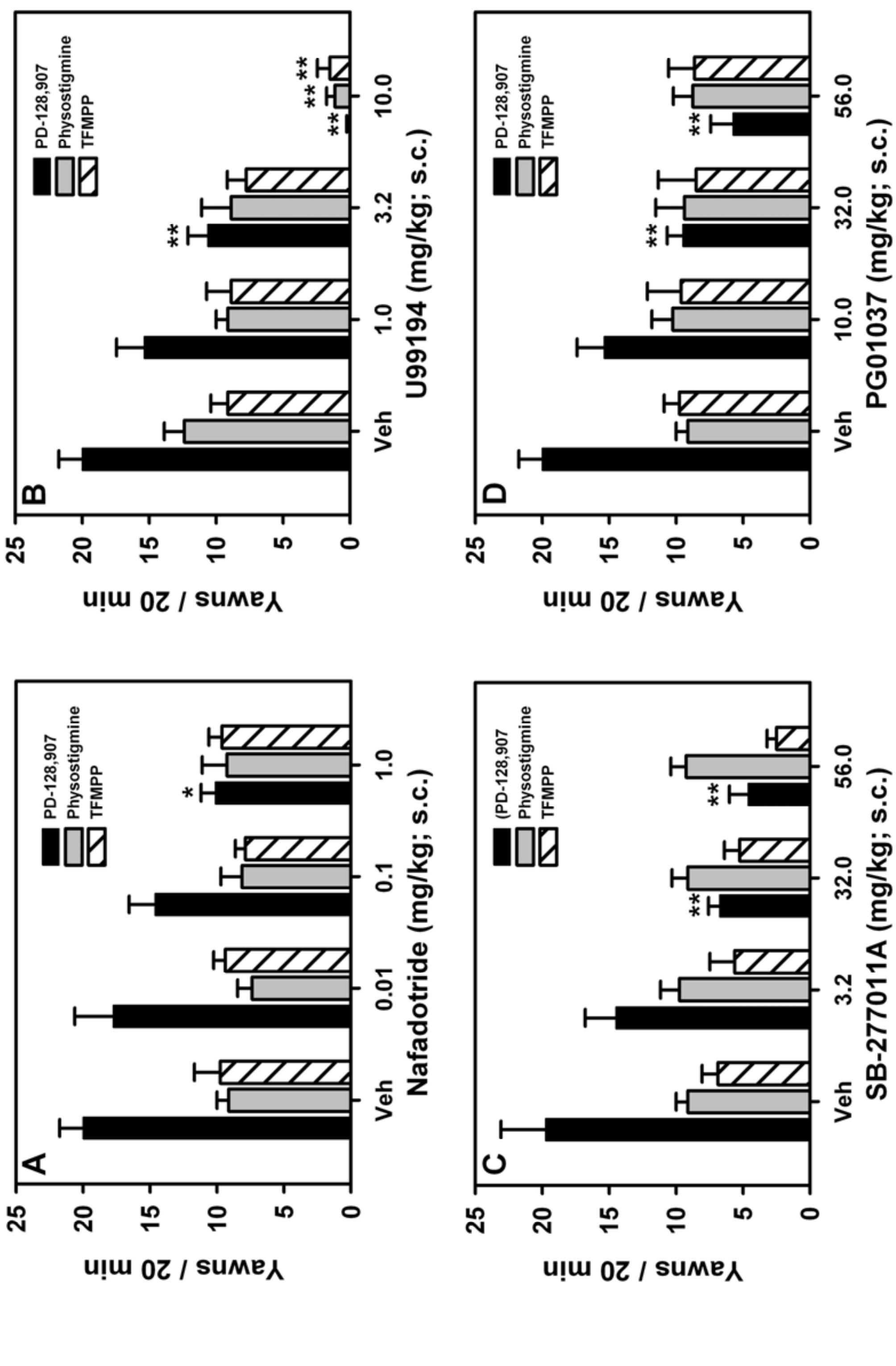


Figure 2.5. Effects of D_3-preferring antagonists on yawning induced by PD-128,907 (0.1 mg/kg), physostigmine (0.1 mg/kg; i.p.) and TFMPP (3.2 mg/kg). A) Nafadotride at doses of 0, 0.01, 0.1, and 1.0 mg/kg; B) U99194 at doses of 0, 1.0, 3.2, and 10.0 mg/kg; C) SB-277011A at doses of 0, 3.2, 32.0, and 56.0 mg/kg; and D) PG01037 at doses of 0, 10.0, 3.2, and 56.0 mg/kg. Data are presented as mean (±SEM), n=8, number of yawns during a 20 minute observation period. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; Significant difference from vehicle-treated rats was determined by one-way repeated-measures ANOVAs with post-hoc Dunnett's tests.

Figure 2.5. Effects of D_3-preferring antagonists on dopaminergic, cholinergic, and serotonergic yawning in rats



CHAPTER III

Yawning and Hypothermia in Rats: Effects of Dopamine D_3 and D_2 Agonists and Antagonists

Introduction

Dopamine D_2 and D_3 receptors are both members of the D_2-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, D_2 receptors are expressed at relatively high levels within cortical, as well as limbic regions, while the D_3 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 D_2 and D_3 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 Trenkwalder, 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 D_3 (e.g., Stemp et al., 2000; Grundt et al., 2005) or D_2 (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 D_2/D_3 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 D_2 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 D_3 selectivity ratios range from 2- to 488-fold selective for the D_3 over D_2 receptor depending upon whether binding affinities from cloned human receptor cell systems or human brain tissue are used to make the determinations (Gerlach et al., 2003; Seeman et al., 2005). Furthermore, *in vitro* binding studies often provide greater affinity and selectivity values than those obtained through functional studies suggesting that differences in D_2 and D_3 efficacy may also greatly influence a ligand's receptor selectivity. For example, in three separate studies which characterized D_2/D_3 agonists based on their binding affinities for the D_2 and D_3 receptors and ability to stimulate mitogenic activity, quinpirole was found to be either 9-, 15- or 36-fold selective for the D_3 over D_2 receptor as determined by radioligand binding, but the D_3 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 (Chio et al., 1994; Pugsley et al., 1995; Sautel et al., 1995a).

The identification of agonists and antagonists highly selective for the D_2 and/or D_3 receptors has been complicated by a lack of well characterized behavioral effects specifically mediated by either the D_2 or D_3 receptor. While D_2/D_3 agonists have been shown to modulate body temperature, locomotor activity, and certain neuroendocrine responses in addition to other behavioral measures (Faunt and Crocker, 1987; Millan et al., 1995b; Depoortere et al., 1996; Smith et al., 1997; Boulay et al., 1999a; Boulay et al., 1999b), 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 D_2 receptor activation, and significant pharmacological evidence for the induction of yawning resulting from agonist activation of the D_3 receptor.

The first indication that D_2/D_3 agonist-induced hypothermia was mediated by the D_2 but not D_3 receptor was the finding that D_3 receptor-deficient mice displayed a normal hypothermic response to D_2/D_3 agonists while the effect was completely absent in D_2 receptor-deficient mice (Boulay et al., 1999a; Boulay et al., 1999b). This was later supported by pharmacologic studies in rats that demonstrated that the D_2-preferring antagonist, L-741,626, produced a dose-dependent inhibition of D_2/D_3 agonist-induced hypothermia, whereas the D_3-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 and Furukawa, 1980), serotonergic (Stancampiano et al., 1994), and dopaminergic (Mogilnicka and Klimek, 1977; Holmgren and Urba-Holmgren, 1980) systems associated with the paraventricular nucleus of the hypothalamus (Argiolas and Melis, 1998). Recently, a specific role for the D_3 receptor in the induction of yawning behavior has also been demonstrated. A series of D_3-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 D_3-preferring antagonists were also shown to selectively inhibit the induction of yawning behavior, while the D_2-preferring antagonist, L-741,626, produced a selective rightward and upward shift in descending limb of the dose-response curve for D_2/D_3 agonist-induced yawning (Collins et al., 2005). Thus, although it has been suggested that the induction of yawning is mediated by activation of the D_2 receptor (Millan et al., 2000), our data indicated that the induction of yawning by D_2/D_3

agonists is mediated by a selective activation of the D_3 receptor while inhibition of yawning behavior at higher doses is a result of a concomitant D_2 receptor activation.

The present studies were aimed at further characterizing the roles of the D_2 and D_3 receptors in the regulation of body temperature and yawning behavior. Thus, a series of D_2-like agonists with a range of reported *in vitro* selectivities for the D_3 over D_2 receptor (pramipexole ≥ PD-128,907 = 7-OH-DPAT > quinpirole = quinelorane >apomorphine > U91356A > sumanirole), as well as two D_4-preferring agonists (ABT-724 and PD-168,077) were assessed for their ability to induce yawning and hypothermia, while a series of D_2/D_3 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 D_2 and D_3 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* D_3 and D_2 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 ºC) 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 x 23 cm x 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.

D_2-Like Agonist-Induced Yawning and Hypothermia: A series of D_2-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 D_2-like Antagonists on Hypothermia and Yawning Behavior: The ability of the D_2 antagonist, L-741,626, and the D_3 antagonist, U99194, to alter hypothermia induced by either D_2/D_3 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 D_2 antagonist, L-741,626, and the D_3 antagonist, PG01037, were assessed for their ability to alter D_2/D_3 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 D_2/D_3 Antagonists on PD-128,907-Induced Yawning Behavior and Sumanirole-Induced Hypothermia: A series of antagonists with varying *in vitro* selectivities for the D_2 and D_3 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 D_3-preferring agonist, PD-128,907. The D_3-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 D_2-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 D_3 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 D_2 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 D_2 and D_3 agonists, respectively.

Results

Agonist-Induced Yawning Behavior and Hypothermia: As shown in Figure 3.1, seven of the eight agonists with significant affinity for the D_3 and D_2

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 D_3-preferring agonists. Unlike the D_3-preferring agonists, the D_2- and D_4-preferring agonists differed in their ability to induce yawning and hypothermia in rats. As shown in Figure 3.1, 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 D_2-preferring agonist. The D_4-preferring agonists, ABT-724 and PD-168,077 (Figure 3.2), 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 D_3 and D_2 receptors.

Table 3.1 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 D_3-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 D_2/D_3 agonists, the currently available *in vitro* data suggests that sumanirole preferentially binds the D_2 over D_3 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 D_3 receptor, and therefore the M.E.D.$_{D3}$ and D_2/D_3 ratio for sumanirole in Table 3.1 are placed in parentheses.

Antagonism of D_2/D_3 Agonist-Induced Yawning and Hypothermia: As shown in Table 3.2, the D_3 antagonist PG01037 and the D_2 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 D_3-preferring agonists, while having no effect on the low levels of yawning observed at the high doses of these agonists. Unlike with the D_3-preferring agonists, the small amount of yawning produced by the D_2-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 D_3 receptors. Pretreatment with the D_2 antagonist L-741,626 (1.0 mg/kg) did not significantly alter induction of yawning by low doses of D_3-preferring agonists, but significantly increased yawning induced by high doses of all D_2/D_3 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 D_3-preferring agonists as well as the D_2-preferring agonist, sumanirole (Table 3.3). Conversely, pretreatment with a behaviorally active dose of the D_3 antagonist, U99194, did not significantly alter the induction of hypothermia resulting from any of the D_2/D_3 agonists tested (Table 3.3).

Antagonism of PD-128,907-Induced Yawning: The left two panels of Figure 3.3 show the effects of the D_3-preferring antagonists on yawning induced by a low and high dose of the D_3-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 3.3). 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 3.3). 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 3.1).

The two left panels of Figure 3.4 demonstrate that, similar to nafadotride, the D_2-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 3.4). Moreover, these effects were observed at doses that did not alter yawning increased by the low dose of PD-128,907 (left panel, Figure 3.4); 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 3.1).

Antagonism of Sumanirole-Induced Hypothermia: The effects of the D_3-preferring antagonists PG01037, SB-277011A, U99194 and nafadotride on sumanirole-induced hypothermia are shown in the right panel of figure 3.3. 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 D_2/D_3 ratios for these antagonists could not be calculated (Table 3.1). A significant and dose-dependent inhibition of sumanirole-induced hypothermia was observed following administration of nafadotride (right panel, Figure 3.3), with an $M.E.D._{D2\ ANT}$ of 0.32 mg/kg (Table 3.1). 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 3.1).

Discussion

The current studies replicate and extend the findings of a previous study that suggested that the induction of yawning by low doses of D_2/D_3 agonists is mediated by the selective activation of the D_3 receptor, whereas the inhibition of yawning occurring at higher doses is mediated by a concomitant activation of the D_2 receptor (Collins et al., 2005). As was demonstrated in the earlier paper, yawning induced by a low dose of the D_3-preferring agonist PD-128,907 was selectively, and dose-dependently inhibited by the D_3 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 D_2 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 D_2/D_3 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

D_2, but not the D_3 or D_4 receptor (Boulay et al., 1999a; Boulay et al., 1999b; Chaperon et al., 2003). Several lines of evidence presented herein support this notion. The selective D_2 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 D_2-preferring antagonists, haloperidol and L-741,626. L-741,626 also inhibited the hypothermic effects of high doses of all of the D_3-preferring agonists in addition to producing dramatic increases in yawning when combined with the same high doses of D_3-preferring agonists. The latter is likely to reflect reversal of the D_2-mediated inhibition of yawning produced at high doses of the agonists, and is consistent with the notion that these antagonists are D_2-selective and that the suppression of yawning and hypothermic effects observed at relatively high doses of D_2/D_3 agonists are D_2 agonist-mediated effects. Importantly, these differential effects of D_3 and D_2 antagonists on yawning induced by low and high doses of D_2/D_3 agonists were observed with all of the D_3-preferring agonists tested in the current study (Table 3.2), 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-1_A agonist, 8-OH-DPAT (Table 3.2) suggesting that these effects are a result of a selective antagonist activity at D_3 and D_2 receptors, respectively.

These *in vivo* measures of selective D_3 (yawning) and D_2 (hypothermia) activation were used to characterize ten D_2-like agonists and six D_2/D_3 antagonists. This extensive evaluation, comparing the potency of each agonist to produce increases in yawning with its potency to produce hypothermia (Table 3.1), indicated that pramipexole was the most selective D_3 agonist, followed by PD-128,907, quinelorane, quinpirole and 7-OH-DPAT with nearly equal D_3 selectivity. Both apomorphine and U91356A were relatively non selective D_2/D_3 agonists, inducing yawning at doses that were only slightly lower than those required to decrease body temperature. Sumanirole was a selective D_2 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 D_3-selective antagonist, PG01037, but was inhibited by the cholinergic antagonist scopolamine and may therefore represent cholinergic rather than D_3 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 D_4-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 D_2 and D_3 receptor agonist activity.

As was seen with the agonists, distinct behavioral profiles emerged for D_3- and D_2-preferring antagonists. Three of the four D_3-preferring antagonists, PG01037, SB-277011A, and U99194 inhibited yawning at doses that did not alter hypothermia suggesting they function as selective D_3 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* D_2/D_3 selectivity ratios were indeterminate other than being slightly greater than 1. Interestingly, nafadotride, which is mildly D_3-preferring *in vitro*, and generally considered to be a D_3-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 D_2 antagonists, haloperidol and L-741,626, than of the other D_3-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 D_2 over D_3 receptor *in vivo*.

Evidence provided in the current, and past (Collins et al., 2005), studies support distinct roles for the D_2 and D_3 receptors mediating the hypothermic and yawning effects of D_2/D_3 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 D_2 and D_3 receptor as it was attenuated by the D_3 antagonists, S33084 and GR218231, as well as the D_2 antagonist, L-741,626. Furthermore, they concluded that 7-OH-DPAT-induced yawning was mediated by the D_2, but not D_3 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 D_3-preferring agonists induce hypothermia, and likewise that relatively large doses of L-741,626 suppress yawning induced by D_3-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 D_2/D_3 agonists and antagonists with respect to their effects on yawning and hypothermia. In the current study, all D_3-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 D_3-selective, dose PD-128,907 was not observed until higher doses. Moreover, in the current study, the D_3 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 D_3 antagonist activity.

While the M.E.D.s 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* D_3 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 D_3 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 D_2 antagonist 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* D_3 selectivity ratios obtained for these agonists and antagonists (Table 3.1) 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 (Chio et al., 1994; Pugsley et al., 1995; Sautel et al., 1995a), 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 D_2-like agonists and antagonists.

To summarize, the results of these studies provide further support for specific roles for the D_3 and D_2 receptors in the mediation of D_2/D_3 agonist-induced yawning behavior and hypothermia, respectively, and demonstrate the usefulness of yawning and hypothermia in the characterization of *in vivo* D3 and D_2 receptor activity. They are the first to provide *in vivo* determinations and comparisons of D_3 receptor selectivities for a series of D_2/D_3 agonists with a range of *in vitro* selectivities for the D_3 or D_2 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 D_3 or D_2 receptors.

Figure 3.1. Dose-response curves for D_2/D_3 agonist-induced yawning (O), 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 3.1. Comparison of yawning and hypothermia induced by D_2-, and D_3-preferring agonists in rats

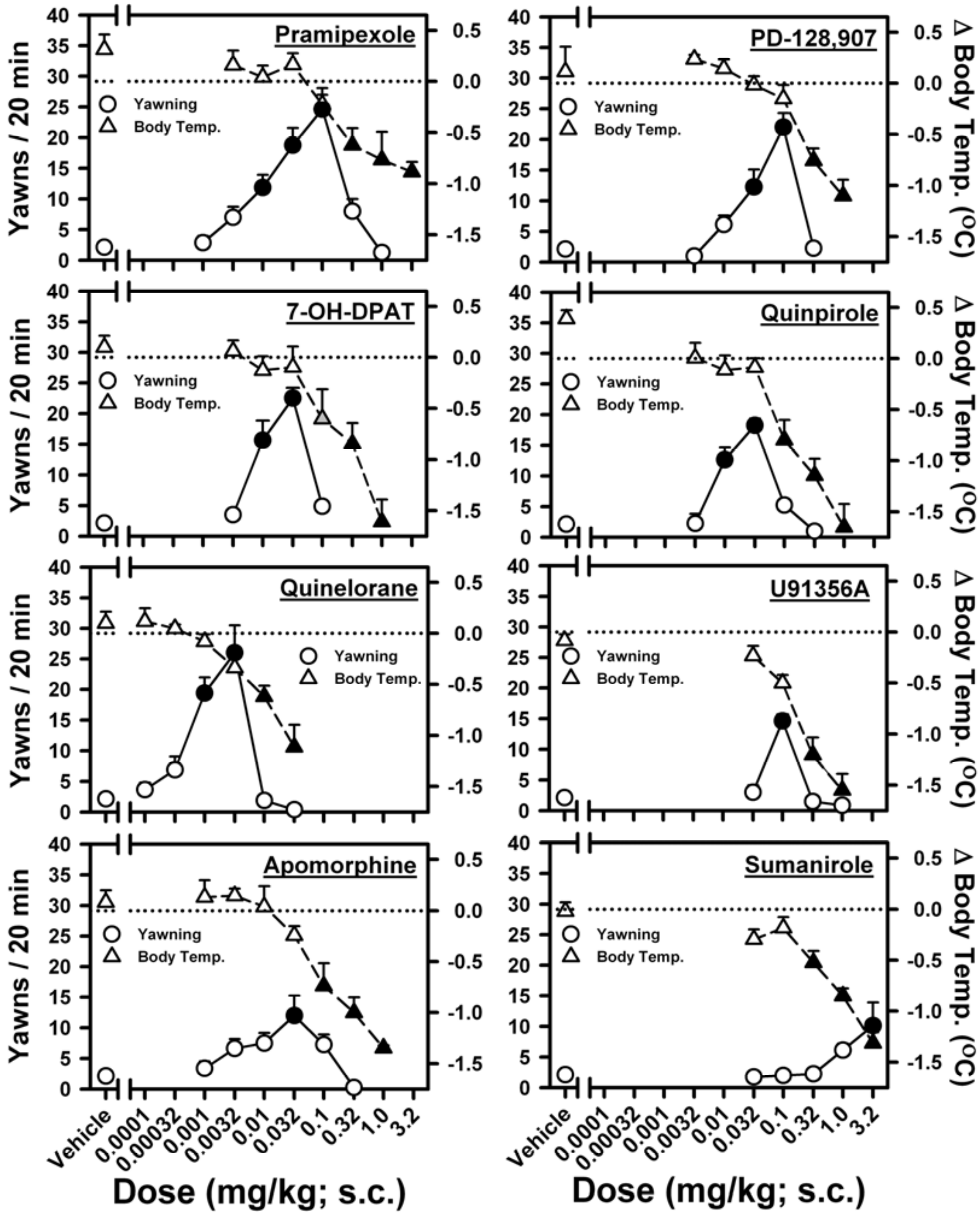


Figure 3.2. Dose-response curves for D_4-preferring agonist-induced yawning (O), and hypothermia (Δ). Characterization of ABT-724 and PD-168,077 was conducted in different groups of rats, with data presented as mean (±SEM), n=8, number of yawns during a 20 minute observation period, and mean (±SEM), 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 3.2. Comparison of yawning and hypothermia induced by D_4-selective agonists in rat

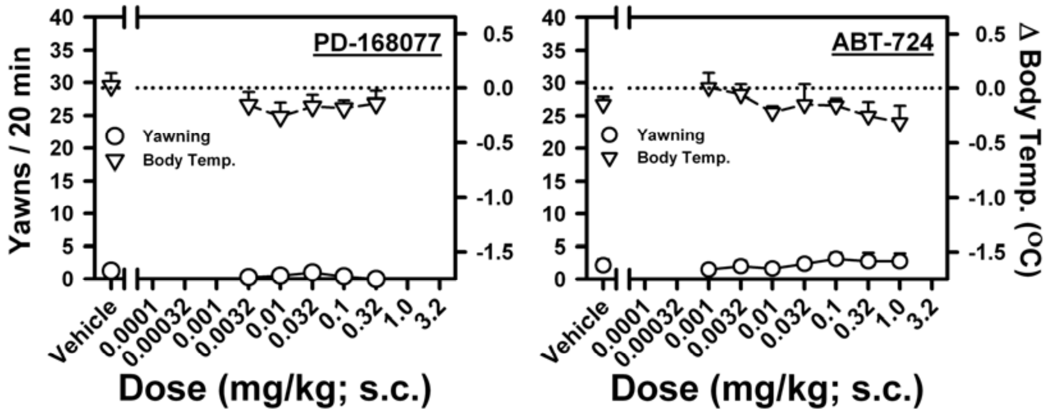


Figure 3.3. Effects of the D_3-preferring antagonists, PG01037 (0, 3.2, 32.0, and 56.0 mg/kg), SB-277011A (0, 3.2, 32.0, and 56.0 mg/kg), U99194 (0, 1.0, 3.2, and 10.0 mg/kg), and nafadotride (0, 0.1, 0.32, and 1.0 mg/kg) on yawning induced by 0.1 mg/kg PD-128,907 (left column), and 0.32 mg/kg PD-128,907 (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 3.3. Effects of D_3-preferring antagonists on PD-128,907-induced yawning and sumanirole-induced hypothermia

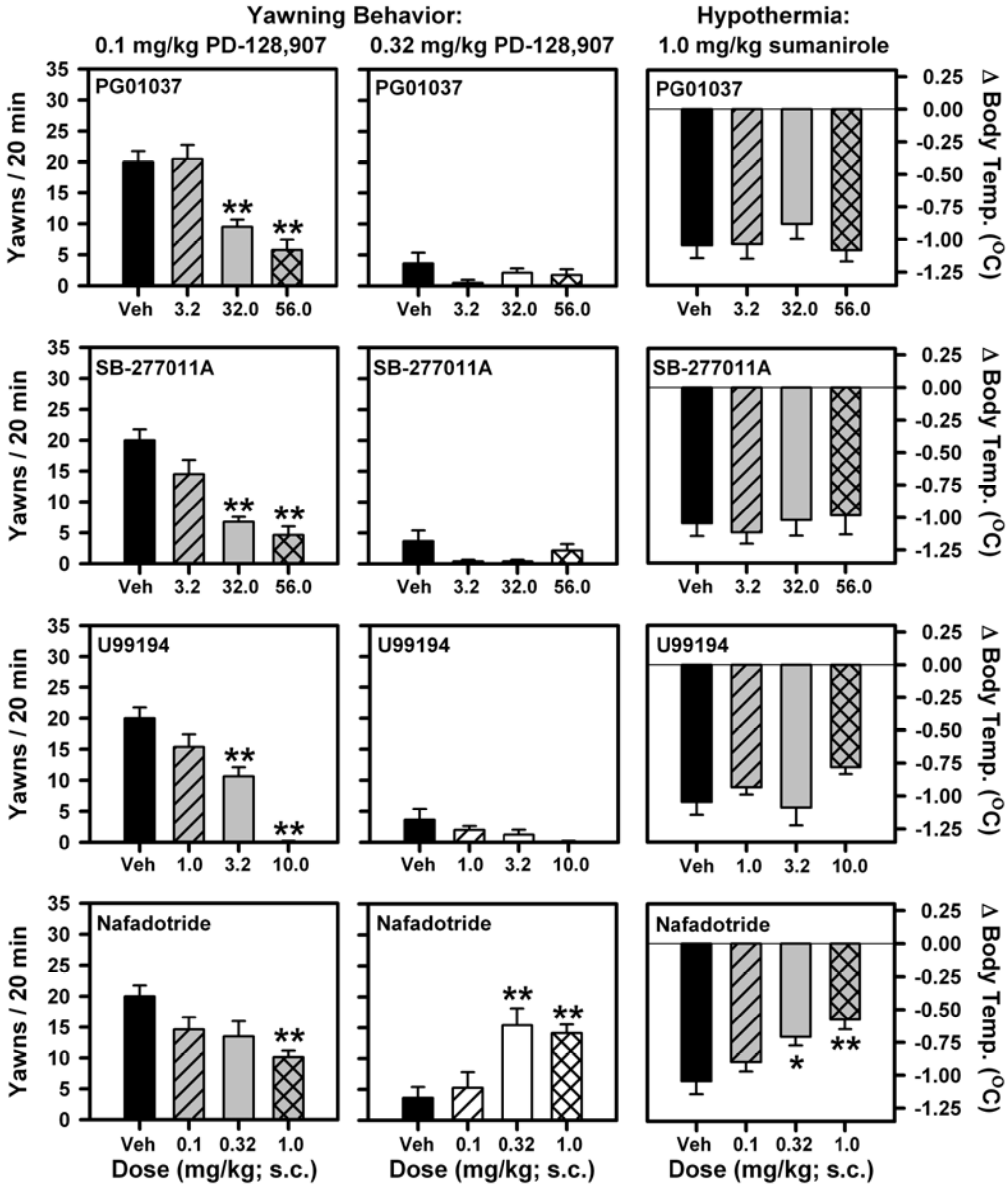


Figure 3.4. Effects of the D_2-preferring antagonists, haloperidol (0, 0.01, 0.032, and 0.1 mg/kg), and L-741,626 (0, 0.32, 1.0 and 3.2 mg/kg) on yawning induced by 0.1 mg/kg PD-128,907 (left column), and 0.32 mg/kg PD-128,907 (center column), or hypothermia induced by 1.0 mg/kg sumanirole (right column). 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.

Figure 3.4. Effects of D_2-preferring and non-selective D_2/D_3 antagonists on PD-128,907-induced yawning and sumanirole-induced hypothermia

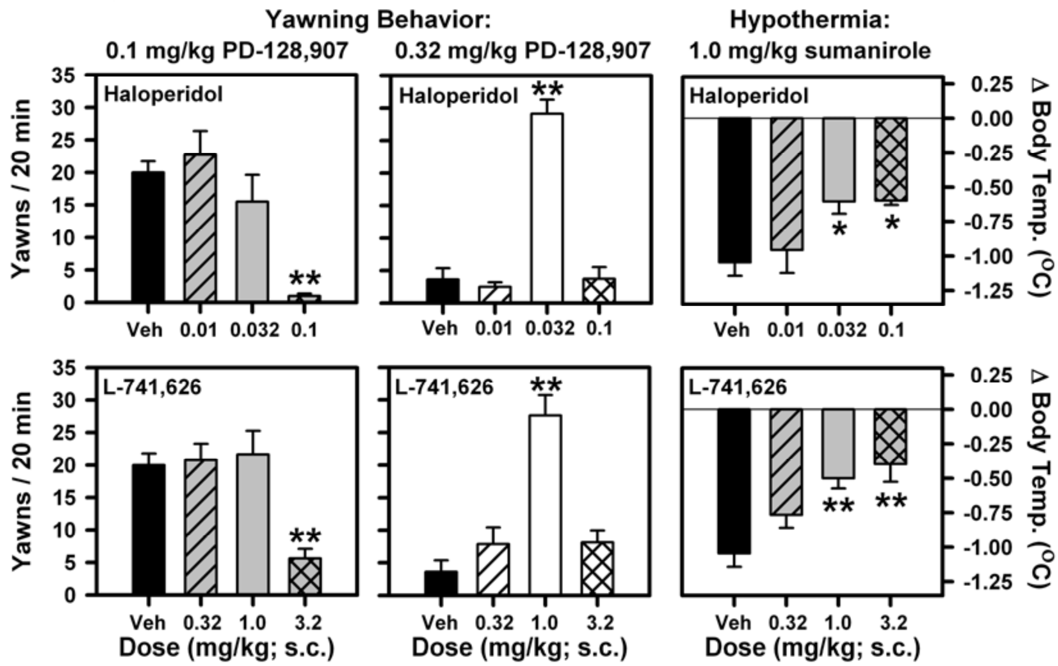


Table 3.1 *in vivo* D_3 selectivity ratios determined from the minimal effective doses for D_2/D_3 agonist-induction, and antagonist-modulation of yawning and hypothermia

Compound	M.E.D. (mg/kg; s.c.) *in vivo* D_2 Hypothermia	*in vivo* D_3 Yawning	*in vivo* D_2/D_3
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 D_3 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* D_3 selectivity ratio could not be determined as compound failed to significantly alter the induction of hypothermia by sumanirole at any dose tested.

Table 3.2 Effects of the D_2 antagonist L-741,626 and the D_3 antagonist PG01037 on D_2/D_3 agonist-induced yawning behavior

	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)**

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 3.3. Effects of the D_2 antagonist L-741,626 and the D_3 antagonist U99194 on D_2/D_3 agonist-induced hypothermia

	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)**
$5\text{-}HT_{1A}$-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.

CHAPTER IV

Pro-erectile Effects of Dopamine D_2-like Agonists are Mediated by the D_3 Receptor in Rats

Introduction

The involvement of dopamine in the regulation of penile erection (PE) has been a long studied phenomenon (Hyyppa et al., 1970), and systemic administration of the non-selective D_2-like agonist, apomorphine, is known to induce PE and yawning in a variety of species including rats (Benassi-Benelli et al., 1979), monkeys (Gisolfi et al., 1980), and man (Lal et al., 1987), suggesting that the receptor regulation of these effects may be similar across species. Several D_3-preferring agonists, including 7-OH-DPAT, pramipexole, and quinpirole (Melis et al., 1987; Ferrari et al., 1993; Ferrari and Giuliani, 1995), have been shown to induce PE over low doses with inhibition of PE occurring at higher doses as has previously been demonstrated for yawning (e.g., Collins et al., 2005; Collins et al., 2007). D_2-like agonist-induced PE and yawning are thought to be centrally mediated as they are inhibited by relatively non-selective, centrally active, D_2-like antagonists such as haloperidol, sulpiride, and clozapine, but not the peripheral D_2-like antagonist domperidone (Benassi-Benelli et al., 1979; Gower et al., 1984; Doherty and Wisler, 1994;

Hsieh et al., 2004). Moreover, a significant body of literature supports a common role for the paraventricular nucleus (PVN) in the induction of PE and yawning by both physiologic and pharmacologic means (e.g.; Argiolas and Melis, 1998; Melis and Argiolas, 1999; Melis and Argiolas, 2003; Argiolas and Melis, 2005), however, the specific receptor(s) mediating the pro-erectile effects of D_2-like agonists are yet to be elucidated.

Recently, a specific role for the D_4 receptor in the induction of PE by D_2-like agonists has been suggested. Dose-dependent increases in the percent incidence of PE were reported following systemic administration of D_4-selective agonists (Hsieh et al., 2004), and further studies have reported similar dose-dependent inductions of PE following systemic (Brioni et al., 2004; Enguehard-Gueiffier et al., 2006; Melis et al., 2006) or intra-PVN (Melis et al., 2005; Melis et al., 2006) administration of a variety of D_4-selective agonists (e.g., ABT-724, CP226269, PD-168,077 and PIP3EA), while the D_4-selective antagonist, L745,870, has been reported to block PD-168,077- and PIP3EA-induced PE (Melis et al., 2005; Enguehard-Gueiffier et al., 2006; Melis et al., 2006). While these findings support a role for the D_4 receptor in the mediation of PE, D_4-selective agonists generally induce fewer erections compared to less selective D_2-like agonists such as apomorphine, and L-745,870 has been shown to be ineffective at altering the induction of PE by apomorphine (Melis et al., 2006), suggesting that other receptor(s) are also involved in the mediation of D_2-like agonist-induced PE. Interestingly, a variety of D_3-preferring agonists (e.g., (+)-

3-PPP, 7-OH-DPAT, pramipexole, quinelorane, and quinpirole) have also been reported to increase PE (Melis et al., 1987; Ferrari et al., 1993; Doherty and Wisler, 1994; Ferrari and Giuliani, 1995) suggesting that D_3 receptors may be involved in the induction of PE by D_2-like agonists.

The current studies were aimed at characterizing the roles of the D_2, D_3, and D_4 receptors in the regulation of D_2-like agonist-induced PE. Thus, *in vitro* binding affinities for a series of D_2-like agonists and antagonists with varying degrees of selectivity for the D_2, D_3, and D_4 receptors were first determined to compare receptor selectivity. Agonists were then assessed for their capacity to induce PE and yawning, while antagonists were assessed for their capacity to alter the induction of PE and yawning by apomorphine and pramipexole. Convergent evidence from the evaluation of the agonists alone, and in combination with antagonists, supports the notion that the induction of PE and yawning by D_2-like agonists are similarly mediated by the D_3 receptor, while the inhibition of PE and yawning observed at higher doses results from a concomitant activation of the D_2 receptor.

Methods

Subjects: Male Wistar rats, 250-350 g, (Harlan; Indianapolis, IN) were housed three to a cage in a temperature and humidity controlled room on a 12-h dark/light cycle with lights on at 7:00 AM. Food and water were freely available; however, no food or water was available during observations. 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 procedures were approved by the University of Michigan Committee on the Use and Care of Animals.

Behavioral observations: On the day of testing rats were transferred from their home cage to a test chamber (48cm x 23cm x 20cm, clear rodent cage with cob bedding), and allowed to habituate for a period of 30 min prior to vehicle or antagonist pretreatment. Following a 30 min pretreatment, one dose of agonist was administered and the total number of yawns and PEs were recorded for a period of 45 min thereafter. Yawning was defined as a prolonged (~1s), wide opening of the mouth followed by a rapid closure, while PE was defined as an emerging, engorged penis usually followed by an upright posture, repeated pelvic thrusts, and genital grooming. All experimental sessions were separated by at least 48 hr to allow for drug washout.

D_2-like agonist-induced yawning and penile erection: The following D_2-like agonists were assessed for their capacity to induce PE and yawning: apomorphine (0.01 - 0.32 mg/kg), pramipexole (0.01 - 1.0 mg/kg), PD-128,907 (0.01 - 0.32 mg/kg), quinpirole (0.0032 - 0.32 mg/kg), sumanirole, (0.1 - 3.2 mg/kg), ABT-724 (0.001 - 0.32 mg/kg), PD-168,077 (0.0032 - 0.32 mg/kg), and PIP3EA (0.0032 - 0.32 mg/kg). All agonists were investigated in separate groups of 8 rats, with each rat receiving each dose of one agonist presented in random order.

Effects of D_2-, D_3-, and D_4-selective antagonists on apomorphine- and pramipexole-induced yawning and penile erection: The following D_2-like antagonists were assessed for their capacity to alter the induction of PE and yawning by apomorphine (0.01 - 0.32 mg/kg) and pramipexole (0.01 - 1.0 mg/kg): PG01037 (32.0 mg/kg), L-741,626 (1.0 mg/kg), and L-745,870 (1.0 mg/kg). PG01037 and L-741,626 was administered as 30 min pretreatments, while L-745,870 was administered 15 min prior to agonist injection. Each antagonist X agonist combination was assessed in separate groups of 8 rats, with each rat receiving all dose combinations in random order.

Effects of D_2-like antagonists on pramipexole-induced yawning and penile erection: The following series of D_2-like antagonists were assessed for their capacity to alter the induction of PE and yawning by pramipexole (0.1 mg/kg): PG01037 (1.0 - 32.0 mg/kg), SB-277011A (1.0 - 32.0 mg/kg), raclopride (0.0032 - 0.1 mg/kg), haloperidol (0.0032 - 0.1 mg/kg), L-741,626 (0.32 - 10.0 mg/kg), Ro-61-6270 (1.0 - 32.0 mg/kg) and L-745,870 (0.32 - 10.0 mg/kg). Each antagonist was assessed in separate groups of 8 rats with each rat receiving all dose combinations, presented in random order.

Drugs: ABT-724 (2-[[4-Pyridin-2-yl)piperazin-1-yl]methyl]-1*H*-benzimidazole) was synthesized by Dr. Kenner Rice (Chemical Biology Research Branch, NIDA, Bethesda, MD). Apomorphine ((*R*)-(-)-5,6,6a,7-Tetrahydro-6-methyl-4*H*-dibenzo[de,g]quinoline-10,11-diol hydrochloride), haloperidol (4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone hydrochloride), PD-128,907 ((*S*)-(+)-(4a*R*,10b*R*)-3,4,4a,10b-Tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano-[4,3-*b*]-1,4-oxazin-9-ol hydrochloride), and quinpirole (trans-(–)-(4aR)-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1*H*-pyrazolo[3,4-*g*]quinoline hydrochloride) were obtained from Sigma-Aldrich (St. Louis, MO). L-741,626 (3-[4-(4-Chlorophenyl)-4-hydroxypiperidin-l-yl]methyl-1*H*-indole), L-745,870 (3-(4-[4-Chlorophenyl]piperazin-1-yl)-methyl-1*H*-pyrrolo[2,3-*b*]pyridine trihydrochloride), PD-168,077 (*N*-(Methyl-4-(2-cyanophenyl)piperazinyl-3-methylbenzamide maleate), and raclopride (3,5-Dichloro-*N*-(1-ethylpyrrolidin-2-ylmethyl)-2-hydroxy-6-methoxybenzamide tartrate salt) were obtained from Tocris (Ellisville, MO). PG01037 (*N*-{4-[4-(2,3-Dichlorophenyl)-piperazin-1-yl]-trans-but-2-enyl}-4-pyridine-2-yl-benzamide hydrochloride) was synthesized by Drs. Amy Newman and Peter Grundt (Medicinal Chemistry Section-NIDA, Baltimore, MD). PIP3EA (2-[4-(2-Methoxyphenyl)piperazin-1-ylmethyl]imidazo[1,2-*a*]pyridine) was synthesized by Drs. Alain Gueiffier and Cécile Enguehard-Gueiffier (Francois-Rabelais Universite, Tours, France). Pramipexole (*N*'-propyl-4,5,6,7-tetrahydrobenzothiazole-2,6-diamine dihydrochloride) and SB-277011A (trans-N-[4-[2-(6-Cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-4-

quinolinecarboxamide) were synthesized by Drs. Shaomeng Wang and Jianyong Chen (University of Michigan, Ann Arbor, MI). Ro 61-6270 (2-amino-benzoic acid-1-benzyl-piperidin-4-yl-ester) was provided by Hoffmann-La Roche (Basel, Switzerland). Sumanirole ((5*R*)-5,6-dihydro-5-(methylamino) 4*H*-imidazo[4,5,1-ij]quinolin-2(1*H*)-one (2*Z*)-2-butenedioate) was synthesized by Drs. Stephen Husbands and Benjamin Greedy (University of Bath, Bath, U.K.). All drugs were dissolved in sterile water with the exceptions of PG01037 and SB-277,011A which were dissolved in 10% β-cyclodextrin, and haloperidol, L-741,626, PD-168,077, and PIP3EA which were dissolved in 5% ethanol and sterile water. All drugs were administered sub-cutaneously in a volume of 0.1 ml/kg, with the exception of L-745,870 which was administered intraperitoneally. The cDNAs for the human dopamine (hD_2, hD_3, and hD_4) receptors were generously provided by Drs. Olivier Civelli (University of California at Irvine), Pierre Sokoloff (INSERM. France) and Dr. Hubert VanTol (University of Toronto, Canada).

Binding Analysis: All K_i values were assessed using membranes prepared from cells recombinantly expressing the hD_2, hD_3 and hD_4 receptors. Ligands were assessed for their capacity to inhibit [^{3}H]PD-128,907 (or [^{3}H]spiperone) binding to the D_3 receptor, or [^{3}H]spiperone binding to the D_2, or D_4 receptor. Membranes for D_2, D_3 and D_4 receptor binding assays were prepared as previously described (Enguehard-Gueiffier et al., 2006) from hD_2-baculovirus-infected insect cells (HighFive Cells, Invitrogen, Carlsbad, CA), or

SH-SY5Y neuroblastoma cells stably expressing either the hD_3 or hD_4 receptor (~1-2 pmol/mg protein). Competitions using [^{3}H]PD-128,907 were performed in a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM $MgSO_4$ and 2 mM $CaCl_2$ with 5 μg of hD_3-SH-SY5Ymembranes in the presence of 2 nM [^{3}H]PD-128,907 and varying concentrations of competing ligands (10^{-11} M to 10^{-4} M, final), while competitions using [^{3}H]spiperone for D_3 (5 μg membrane), D_2 (5 μg membrane), and D_4 (10 μg membrane) receptors were performed in 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA, 2 mM $MgSO_4$ and 2 mM $CaCl_2$ with 2 nM (D_3) or 200 pM (D_2 and D_4) [^{3}H]spiperone (final volume of 500 μl) in the presence of varying concentrations of competing ligands (10^{-11} M to 10^{-4} M, final). Radioligand binding assays were performed at room temperature in 96-well microtiter plates, and filtered onto GF/B filter plates with radioactivity detected by liquid scintillation counting on a TopCount counter (Perkin-Elmer, Waltham, MA). All K_i values were determined from the IC_{50} values derived by non-linear fitting analysis, and the K_d values for [^{3}H]spiperone on the D_2 and D_4 receptor and [^{3}H]PD128-907 on the D_3 receptors (not shown), according to Cheng-Prusoff (Cheng and Prusoff, 1973).

Data analysis: Radioligand binding data were analyzed using a non-linear regression fitting program and analyzed for one-or two-site inhibition curves (GraphPad Prism, San Diego, CA). All yawning and PE studies were conducted with 8 rats per group with results expressed as the mean number of yawns or PE observed over 45 min ± standard error of the mean (S.E.M.).

Percent incidence represents the number of rats displaying at least one PE during the 45 min observation period. Significant effects of agonists on the induction of PE, or antagonists on agonist-induced PE were determined using Mann-Whitney U-Tests (GraphPad Prism). One-way, repeated-measures ANOVA with post-hoc Dunnett's tests was used to determine significant levels of agonist-induced yawning (GraphPad Prism), while significant effects of antagonists on apomorphine-, and pramipexole-induced yawning were determined using two-way ANOVA with post-hoc Bonferroni tests (SPSS, SPSS Inc., Chicago, IL). One-way repeated-measures ANOVA with post-hoc Dunnett's tests were used to determine significant effects of antagonists on pramipexole-induced yawning. (GraphPad Prism).

Results

Since a comparison of binding affinities of the ligands used in these studies at the D_2, D_3, and D_4 receptors has not been previously reported in a single study, the binding potencies of each compound against recombinantly-expressed human hD_2, hD_3, and hD_4 receptors were directly compared using radioligand filter binding assays to allow for a proper comparison of the receptor subtype selectivities of the D_2-like ligands used in these studies. The capacity of all of the agonists and antagonists to displace the antagonist, $[^3H]$spiperone, was assessed for each receptor subtype, while displacement of the D_3-preferring agonist, $[^3H]$PD-128,907 was also assessed for the D_3

receptor subtype. Most ligands displaced radioactive probes with a single phase inhibition, consistent with a one-site model; only agonist binding to D_2 receptors displayed biphasic inhibition curves (composed of a low affinity state and a guanine nucleotide-sensitive high affinity state). Binding affinities and selectivity ratios for ligands binding to the D_2 and D_3 receptors (D_2/D_3) and D_4 and D_3 receptors (D_4/D_3) are shown in Tables 4.1 and 4.2; note that the more relevant comparisons with the D_{2high} state and D_3 receptors (D_{2high}/D_3) are also shown. The K_i's obtained in this studies are generally consistent with those reported in several previous studies, though the absence of good correspondence with *in vivo* activity is duly noted as previously described (e.g., Levant, 1997).

D_2-like agonist-induced yawning and penile erection: Dose-dependent increases in PE and yawning were observed for the non-selective D_2-like agonist, apomorphine, as well as the D_3-preferring agonists, PD-128,907, pramipexole, and quinpirole, while inhibition of both responses occurred at higher doses resulting in inverted U-shaped dose-response curves for PE and yawning (Figure 4.1). Peak levels of PE and yawning were observed at the same dose for apomorphine (0.1 mg/kg), pramipexole (0.1 mg/kg), and PD-128,907 (0.1 mg/kg), while doses of 0.032 and 0.1 mg/kg quinpirole induced peak levels of yawning and PE respectively. Apomorphine, pramipexole, and PD-128,907 induced at least one PE over the 45 min in 87.5% of rats, while the maximal percent incidence of PE for quinpirole was 75%. None of the D_4-selective agonists induced significant levels of PE or yawning (Figure 4.1). PIP3EA induced at least one PE in 50% of rats at a dose of 0.1 mg/kg; the maximal percent incidence of PE for PD-168,077 and ABT-724 was 25%. While significant levels of yawning were observed with the D_2-preferring agonist, sumanirole, PE was not induced (Figure 4.1).

D_3-, D_2-, and D_4-selective antagonism of apomorphine- and pramipexole-induced yawning and erection: The effects of the D_3-selective antagonist, PG01037, the D_2-selective antagonist, L-741,626, and the D_4-selective antagonist, L-745,870 on apomorphine- and pramipexole-induced PE and yawning are shown in figure 4.2. Significant inhibition of the induction of both PE and yawning by apomorphine and pramipexole was observed following a dose of 32.0 mg/kg PG01037; no effect on the inhibition of PE or yawning observed at higher doses was observed (Figure 4.2A-D). PG01037 also reduced the maximal percent incidence of PE for APO from 87.5% to 12.5%, and from 87.5% to 25% for pramipexole (Figure 4.2E-F). Unlike with PG01037, the D_2-selective antagonist, L-741,626 (1.0 mg/kg) selectively reversed the inhibition of PE and yawning observed at higher doses of apomorphine and pramipexole while having no effect on the induction of yawning at lower doses (Figure 4.2G-J). Pretreatment with L-741,626 not only increased the maximal number of PEs and yawns observed, but also shifted the peaks of the PE and yawning dose-response curves for apomorphine and pramipexole ½ log unit to the right. L-741,626 also shifted the descending limb of the dose-response curves for the percent incidence of PE for apomorphine and pramipexole resulting in 100% of rats exhibiting at least one PE at doses of 0.1 and 0.32 mg/kg (Figure 4.2K and 4.2L). When given at a behaviorally active dose of 1.0 mg/kg (Enguehard-Gueiffier et al., 2006), L-745,870 failed to modify apomorphine- or pramipexole-induced PE or yawning, and furthermore, did not alter the percent incidence of PE for either apomorphine or pramipexole (Figure 4.2M-R).

D_3, D_2, and D_4 antagonism of pramipexole-induced yawning and erection: The effects of a series of D_2-like antagonists, with varying degrees of selectivity for the D_2, D_3, and D_4 receptors, on PE and yawning induced by the maximally effective dose of pramipexole (0.1 mg/kg) are shown in figure 4.3. Dose-dependent inhibition of pramipexole-induced PE and yawning was observed with both of the D_3-selective antagonists, PG01037 and SB-277011A (Figure 4.3A-B), however, there were slight differences in the relative potencies with PG01037 inhibiting PE at a dose (3.2 mg/kg) ½ log unit lower than that required to inhibit yawning (10.0 mg/kg), while SB-277011A was equipotent at inhibiting the induction of yawning and PE (10.0 mg/kg). Similar to SB-277,011A, inhibition of pramipexole-induced yawning and PE was observed at the same dose (0.032 mg/kg) for the non-selective D_2/D_3 antagonist, raclopride (Figure 4.3C), while the relatively non-selective D_2-like antagonist, haloperidol, and the D_2-selective antagonist, L-741,626, produced a dose-dependent inhibition of pramipexole-induced PE and yawning with a significant inhibition of yawning observed at a dose ½ log unit lower than was required to inhibit the induction of PE (Figure 4.3D-E). Unlike all other D_2-like antagonists tested, the D_4-selective antagonists, L-745,870 (Figure 3F) and Ro 61-6270 (Figure 4.3G), did not alter the induction of either PE or yawning by pramipexole, although a slight, but not significant, reduction of pramipexole-induced PE was observed following a dose of 10.0 mg/kg L-745,870.

Discussion

These studies were aimed at characterizing a series of D_2-like agonists and antagonists, with varying degrees of selectivity for the D_2, D_3, and D_4 receptors, with respect to their capacity to modulate the induction of PE in rats. Convergent evidence from the evaluation of the effects of the agonists alone, and in combination with D_2-, D_3-, and D_4-selective antagonists suggests that the induction of PE is mediated by activation of the D_3 receptor, while the inhibition of PE observed at higher doses results from the concomitant activation of the D_2 receptor. These studies also confirm previous reports (Collins et al., 2005; Collins et al., 2007) suggesting a similar role for the D_3 (induction) and D_2 (inhibition) with respect to D_2-like agonist induced yawning behavior. However, a role for the D_4 receptor in the mediation of D_2-like agonist-induced PE was not supported.

In agreement with previous reports, apomorphine, pramipexole, and quinpirole induced PE and yawning with inverted U-shaped dose-response curves, and 75 to 87.5% of rats displaying at least one PE at the peak dose, however, these are the first studies to report a similar capacity of the D_3-preferring agonist, PD-128,907, to induce PE. Moreover, increases in yawning and PE were observed over a similar range of doses for all agonists even though large differences exist between these agonists with respect to their *in vitro* selectivity for D_3 compared to D_4 receptors (e.g., apomorphine $D_4/D_3 \approx 0.05$ and PD-128,907 $D_4/D_3 \approx 1280$; Table 4.1), suggesting that their capacity to induce PE is related to their activity at the D_3, but not D_4 receptor. In agreement with this notion, but contrary to previous findings (Brioni et al., 2004; Melis et al., 2005; Enguehard-Gueiffier et al., 2006), the highly selective D_4 agonists all failed to induce significant levels of PE. Although the current studies were unable to confirm the pro-erectile effects of D_4 agonists, it should be noted that the total number of PEs observed for apomorphine, quinpirole, and pramipexole in the current study was lower than previous reports (e.g., Melis et al., 2006) suggesting differences in procedure may have affected the PE response. However, as the percent incidence of PE for apomorphine and quinpirole was similar to previous reports (e.g., Hsieh et al., 2004), any potential differences in procedure only affected the magnitude of the PE response, but not the capacity of the agonists to induce PE.

Furthermore, although D_4-selective agonists have been reported to induce PE, they have generally been shown to be less effective than other D_2-like agonists, such as apomorphine (Melis et al., 2005; Melis et al., 2006), suggesting that these compounds may be functioning as partial agonists, although as increases in extracellular dopamine have been shown to correspond to the induction of PE resulting from the non-contact exposure of a receptive female (Melis et al., 2003), D_4 agonists may be potentiating the pro-erectile effects of other receptor subtypes activated by endogenous dopamine. Interestingly, similar increases in dopamine have also been reported with exposure to novelty (Feenstra et al., 2000; Legault and Wise, 2001; van der Elst et al., 2005), and light-dark transitions (Smith et al., 1992) suggesting that procedural differences such as lighting conditions (Brioni et al., 2004), or experimental history (Brioni et al., 2004; Enguehard-Gueiffier et al., 2006; Melis et al., 2006) may be sufficient to alter the effects of D_2-like agonist. In fact, light-dark transitions have been shown to increase both spontaneous (Anias et al., 1984) and apomorphine-induced yawning (Nasello et al., 1995), suggesting light-dark transitions can enhance D_3-mediated behaviors. Thus, it is possible that the reported pro-erectile effects of D_4-selective agonists may have resulted from a combined effect of an increased endogenous activation of D_3 receptors, and a potentiation this effect by agonist activation of the D_4 receptor.

Specific roles for the D_3 and D_2, but not D_4 receptor, in the mediation of D_2-like agonist-induced PE is further supported by the effects of D_2-, D_3-, and

D_4-selective antagonists on apomorphine- and pramipexole-induced PE and yawning. When given at behaviorally active doses (Collins et al., 2005; Enguehard-Gueiffier et al., 2006; Collins et al., 2007), PG01037, and L-741,626 differentially effected apomorphine- and pramipexole-induced PE and yawning, while no effect of L-745,870 on the induction or inhibition of PE or yawning was observed. Similarly, to the effects of D_3 and D_2 antagonists on yawning, PG01037 produced a selective rightward and/or downward shift of the ascending limb, while L-741,626 produced a selective rightward shift of the descending limb of the PE dose-response curves for apomorphine and pramipexole with respect to both the absolute number or PEs observed, as well as the percent incidence of PE. Together with the finding that yawning and PE were induced over similar ranges of doses, these results support the notion that the induction of PE by D_2-like agonists is mediated by the activation of the D_3 receptor, while the inhibition of PE observed at higher doses results from a concomitant activation of the D_2 receptor, as has been previously reported for yawning (Collins et al., 2005; Collins et al., 2007).

This general notion is further supported by the dose-response analysis of a series of D_2-like antagonists on pramipexole-induced PE and yawning. Dose-dependent inhibition of pramipexole-induced PE was observed following pretreatment with D_3-selecitve (PG01037 and SB-277011A), non-selective D_2/D_3 (raclopride), non-selective D_2-like (haloperidol), and D_2-selective (L-741,626) antagonists, an effect that was correlated with the inhibition of yawning, but was not observed with either of the D_4-selective antagonists (L-745,870 and Ro 61-6270). Furthermore, as was seen with the capacity of D_2-like agonists to induce PE and yawning, the potencies of D_2-like antagonists to inhibit PE was similar to their potencies to inhibit yawning regardless of the fact that large differences exist with respect to their *in vitro* selectivity for D_3 compared to D_4 receptors (e.g., PG01037 $D_4/D_3 \approx 1.3 \times 10^{04}$, raclopride $D_4/D_3 \approx$ 64, and haloperidol $D_4/D_3 \approx 0.1$; Table 4.2), while antagonists highly selective for the D_4 compared to D_3 receptors (e.g., L-745,870 $D_4/D_3 \approx 1.7 \times 10^{-04}$ and Ro 61-6270 $D_4/D_3 \approx 9.1 \times 10^{-05}$; Table 4.2) failed to alter pramipexole-induced PE or yawning. While Ro 61-6270 has not been extensively characterized (Clifford and Waddington, 2000), L-745,870 has been shown to possess favorable pharmacokinetics (0.3 mg/kg; p.o. is thought to be sufficient to occupy ~90% of D_4 receptors; (Patel et al., 1997), and has been shown to inhibit PD-168,077- and PIP3EA-induced PE at a dose of 1.0 mg/kg (Enguehard-Gueiffier et al., 2006; Melis et al., 2006), suggesting that the range of doses used in the current studies were sufficient to block D_4 receptors. Together with previous reports that L-745,870 was unable to alter apomorphine-induced PE (Melis et al., 2006), the current studies suggest that the pro-erectile effects of D_2-like agonists (e.g., apomorphine and pramipexole) are mediated by activation of the D_3, but not D_4 receptor. Inferences with regard to the receptors mediating the pro-erectile effects of D_4-selective agonists could not be made as all D_4-selective agonists failed to induce PE in the current studies.

To summarize, a series of D_2-like agonists with varying selectivities for the D_2, D_3, or D_4 receptors, alone, and in combination with a series of D_2-like antagonists with varying selectivities for the D_2, D_3, or D_4 receptors were assessed for their capacity to induce PE and yawning in rats. Similar to apomorphine, all D_3-preferring agonists induced dose-dependent increases in PE and yawning over a similar range of low doses, while inhibition of PE and yawning occurred at higher doses, while all D_4-selective agonists failed to induce either PE or yawning at any dose tested. The D_3-selective antagonist, PG01037, and D_2-selective antagonist, L-741,626, had similar effects on apomorphine- and pramipexole-induced PE and yawning, with PG01037 selectively inhibiting the induction, and L-741,626 selectively reversing the inhibition of PE and yawning observed at higher doses. Furthermore, a series of D_2-like antagonists with a wide range of selectivities for the D_3 and D_2 receptors dose-dependent inhibited pramipexole-induced PE and yawning with similar potencies, while D_4-selective antagonists were ineffective. In conclusion, these studies provide convergent evidence in support of a role for the D_3 receptor in the induction of PE by D_2-like agonists, with the inhibition of PE observed at higher doses resulting from the concomitant activation of the D_2 receptor.

Figure 4.1. Dose-response curves for D_2-like agonist-induced PE and yawning. Characterization of PE and yawning induced by A) apomorphine; B) pramipexole; C) quinpirole; D) PD-128,907; F) ABT-724; G) PD-168,077; H) PIP3EA; and I) sumanirole was conducted in separate groups of rats with data presented as mean (±SEM), n=8, number of PEs and yawns observed in 45 min. E and J) Percent of rats displaying at least one PE over 45 min. *, $p<0.05$; **, $p<0.01$. Significant differences in agonist-induced yawning as determined using one-way, repeated-measures ANOVA with post-hoc Dunnett's tests and, +, $p<0.05$; ++, $p<0.01$; agonist-induced PE as determined by Mann-Whitney U-Test compared to vehicle treated animals.

Figure 4.1. Comparison of yawning and penile erection induced by D_2-, D_3-, and D_4-preferring agonists in rats

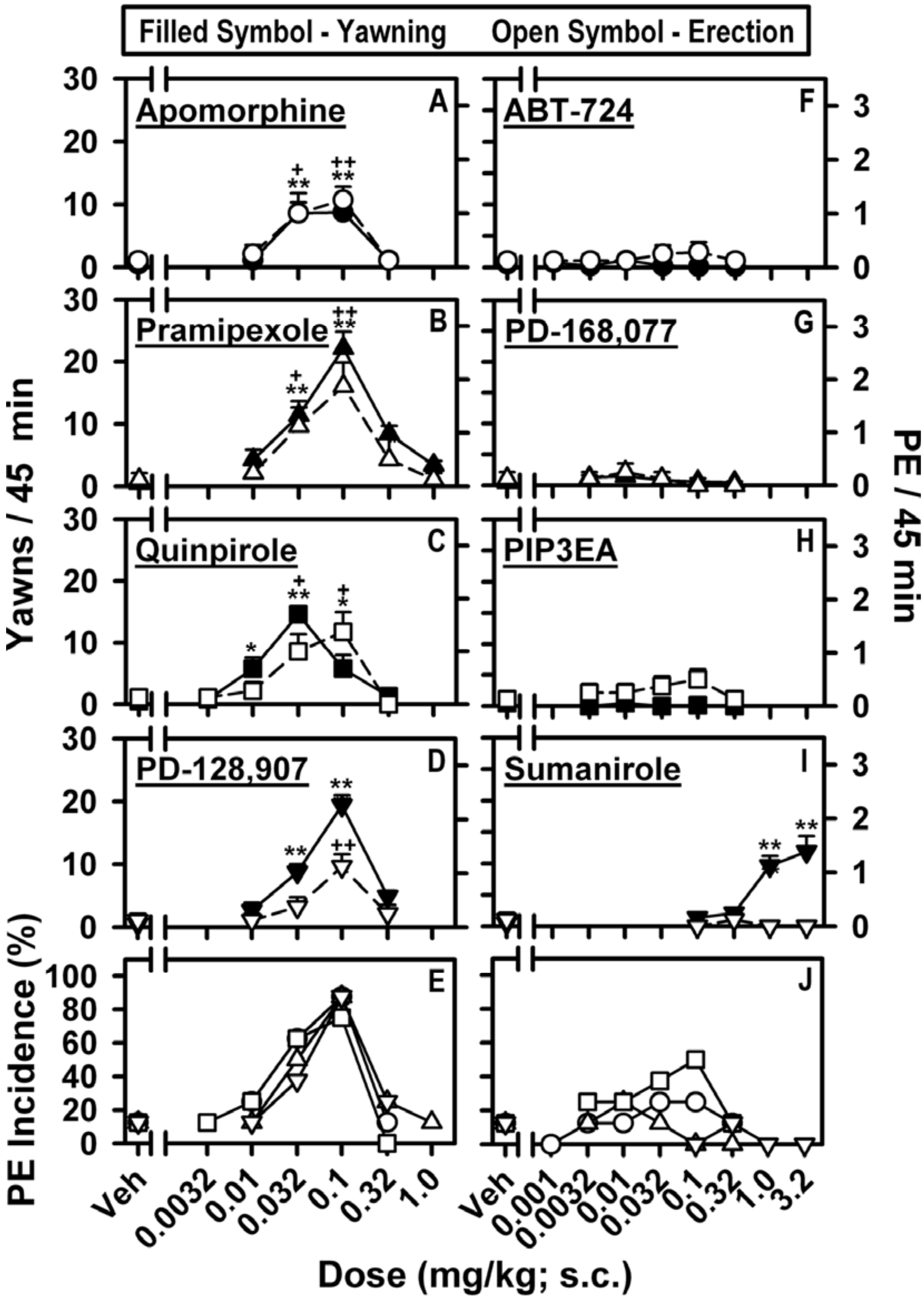


Figure 4.2. D_3-, D_2-, and D_4-selective antagonists on apomorphine- and pramipexole-induced PE and yawning. Effects of the D_3-selective antagonist PG01037 (32.0 mg/kg) on apomorphine- and pramipexole-induced A and B) yawning; C and D) PE; E and F) percent incidence of PE. Effects of the D_2-selective antagonist L-741,626 (1.0 mg/kg) on apomorphine- and pramipexole-induced G and H) yawning; I and J) PE; K and L) percent incidence of PE. Effects of the D_4-selective antagonist L-745,870 (1.0 mg/kg) on apomorphine- and pramipexole-induced M and N) yawning; O and P) PE; Q and R) percent incidence of PE. Data are presented as mean (±SEM), n=8, number of PEs and yawns observed in 45 min. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Significant effect of antagonist on agonist-induced yawning as determined by a two-way ANOVA with post-hoc Bonferroni tests. +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$. Significant effect of antagonist on agonist-induced PE as determined by Mann-Whitney U-Test.

Figure 4.2. Effects of D_2-, D_3-, and D_4-selective antagonists on apomorphine- and pramipexole-induced yawning and penile erection in rats

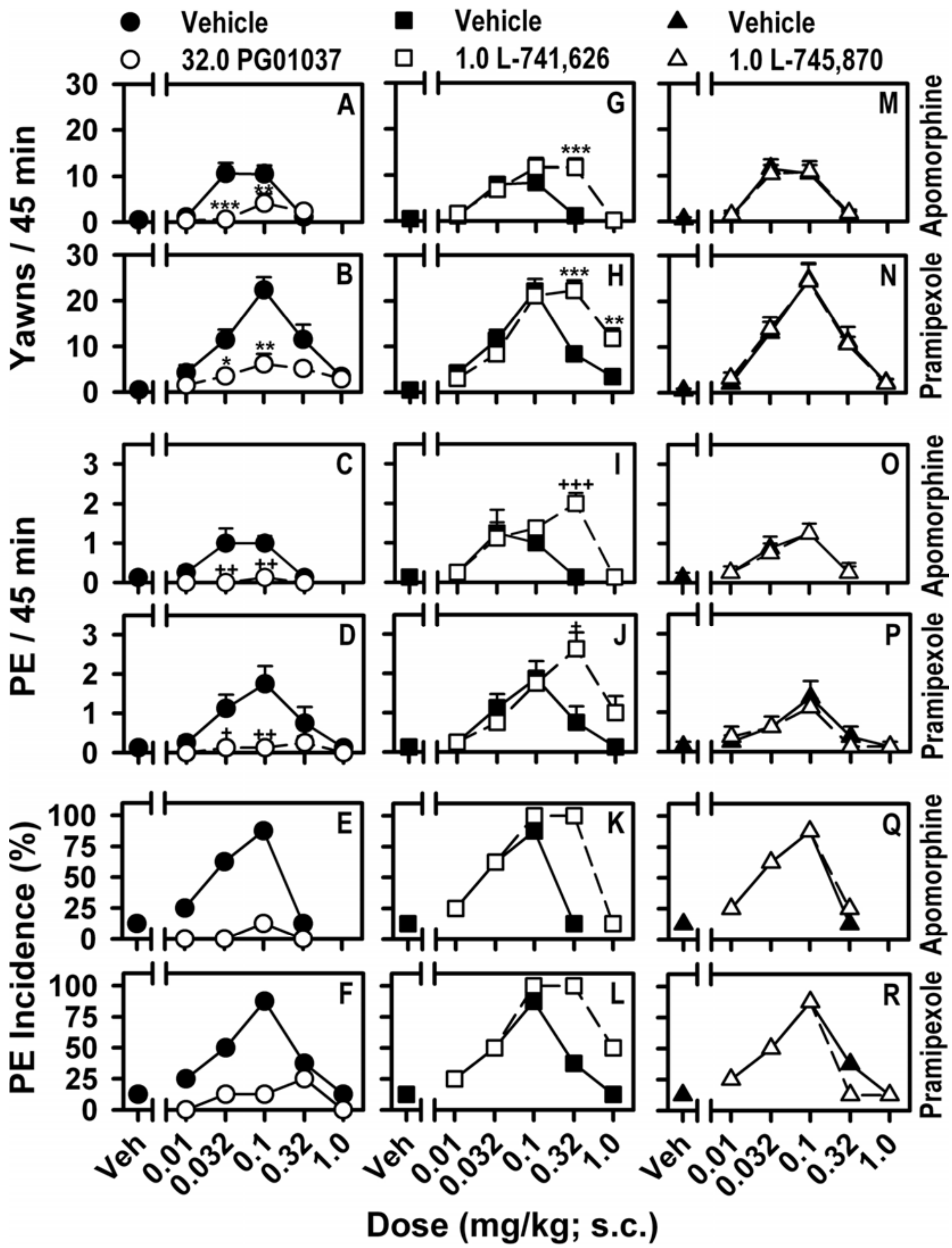


Figure 4.3. Effects of a series of D_2-like antagonists with a range of selectivities for the D_3, D_2, and D_4 receptors on PE and yawning induced by 0.1 mg/kg pramipexole. Effects of the D_3-selective antagonists A) PG01037 (1.0-32.0 mg/kg); and B) SB-277011A (1.0-32.0 mg/kg); the non-selective D_2/D_3 antagonist C) raclopride (0.0032-0.1 mg/kg); the non-selective D_2-like antagonist D) haloperidol (0.0032-0.1 mg/kg); the D_2-selective antagonist E) L-741,626 (0.32-10.0 mg/kg); and the D_4-selective antagonists f) L-745,870 (0.32-10.0 mg/kg); and G) Ro 61-6270 (1.0-32.0 mg/kg). *, $p<0.05$; **, $p<0.01$. One-way repeated-measures ANOVAs with post-hoc Dunnett's tests were used to determine significant effects of antagonists on pramipexole-induced yawning and +, $p<0.05$; ++, $p<0.01$; Mann-Whitney U-Tests were used to determine significant effects of antagonists on pramipexole-induced PE.

Figure 4.3 Effects of D_2-, D_3-, D_4-selective, and non-selective D_2-like antagonists on pramipexole-induced yawning and penile erection in rats

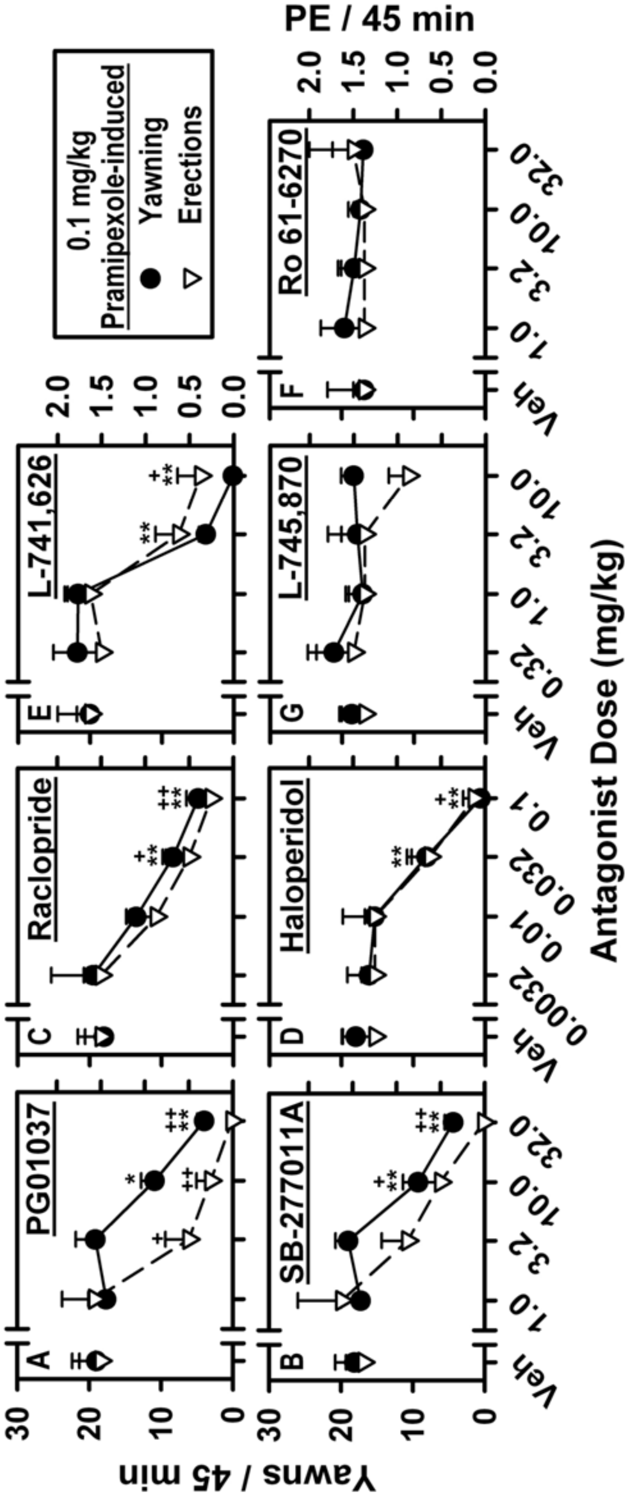


Table 4.1. *In vitro* binding affinities and selectivity ratios at D_2, D_3, and D_4 receptors for D_2-like agonists

Agonist	hD_2 [^{3}H]Spip (nM)	hD_2 [^{3}H]Spip K_{high} (nM)	hD_2 [^{3}H]Spip K_{low} (nM)	hD_3 [^{3}H]PD-128,907 (nM)	hD_3 [^{3}H]Spip (nM)	hD_4 [^{3}H]Spip (nM)	D_2/D_3 † Ratio	D_{2high}/D_3 † Ratio	D_4/D_3 † Ratio
pramipexole	>10,000	*n.d.* ¶	*n.d.* ¶	0.46	10.2	194	*n.a.* [a]	*n.a.* [a]	422
PD-128,907	2840	5.7	>10,000	1.9	9.7	2430	1495	3	1280
quinpirole	1520	2	2070	6	9.4	109	253	0.3	18.2
apomorphine	45	45	1280	75	231	3.4	0.6	0.6	0.05
ABT-724	>10,000	*n.d.* ¶	*n.d.* ¶	>10,000	947	58	*n.a.* [a]	*n.a.* [a]	*n.a.* [a]
PD-168,077	4250	*n.d.* ¶	*n.d.* ¶	1400	726	23	3.04	*n.a.* [a]	0.02
PIP3EA	32	1.74	950	1720	1910	3.7	0.02	1.0×10^{-03}	2.2×10^{-03}
sumanirole	144	0.15	256	613	493	>10,000	0.2	2.4×10^{-04}	*n.a.* [a]

† *Selectivity ratio based on Ki values from D2, D2(high) and D4 using [^{3} H]Spiperone and values from D3 using [3H]PD-128,907*

¶ *Not Determined*

[a] *Selectivity ratio could not be calculated*

Table 4.2. *In vitro* binding affinities and selectivity ratios at D_2, D_3, and D_4 receptors for D_2-like antagonists

Antagonist	hD_2 [^{3}H]Spip (nM)	hD_2 [^{3}H]Spip K_{high} (nM)	hD_2 [^{3}H]Spip K_{low} (nM)	hD_3 [^{3}H]PD-128,907 (nM)	hD_3 [^{3}H]Spip (nM)	hD_4 [^{3}H]Spip (nM)	D_2/D_3 † Ratio	D_{2high}/D_3 † Ratio	D_4/D_3 † Ratio
PG01037	52	*n.d.* ¶	*n.d.* ¶	0.057	0.032	760	912	*n.a.* [a]	1.3×10^{04}
SB-277011A	527	*n.d.* ¶	*n.d.* ¶	78	74	3600	6.8	*n.a.* [a]	46
raclopride	2.2	*n.d.* ¶	*n.d.* ¶	79	8.8	5030	0.03	*n.a.* [a]	64
haloperidol	3	*n.d.* ¶	*n.d.* ¶	16	33	2.1	0.2	*n.a.* [a]	0.1
L-741,626	18.1	*n.d.* ¶	*n.d.* ¶	604	271	260	0.03	*n.a.* [a]	0.4
L-745,870	3600	*n.d.* ¶	*n.d.* ¶	3020	872	0.5	1.2	*n.a.* [a]	1.7×10^{-04}
Ro 61-6270	1450	*n.d.* ¶	*n.d.* ¶	5470	793	0.5	0.3	*n.a.* [a]	9.1×10^{-05}

† *Selectivity ratio based on Ki values from D2, D2(high) and D4 using [^{3}H]Spiperone and values from D3 using [3H]PD-128,907*

¶ *Not Determined*

[a] *Selectivity ratio could not be calculated*

CHAPTER V

Conclusions

The evaluation of unconditioned, elicited behavioral effects has been an important and long-used method for characterizing potential agonist and/or antagonist activity of novel ligands acting on a variety of neurotransmitter systems. For example, while drugs that increase synaptic serotonin levels were known to induce a behavioral syndrome consisting of behaviors such as resting tremor, head-twitch, hyperactivity, lower lip retraction, salivation, head weaving, and forepaw treading (e.g., Chessin et al., 1957; Udenfriend et al., 1957; Hess and Doepfner, 1961), it was not until the specific receptors mediating the individual behaviors within this syndrome were defined that the head-twitch response (Corne et al., 1963; Colpaert and Janssen, 1983; Green et al., 1983) and lower lip retraction (Berendsen et al., 1989; Koek et al., 1998) became useful tools for the evaluation of agonist and antagonist activity at the 5-HT_2 and 5-HT_{1A} receptors, respectfully.

Similarly, although agonists acting at D_2-like receptors have long been reported to induce a variety of behavioral effects including yawning (Mogilnicka and Klimek, 1977), stretching (Baggio and Ferrari, 1983), sniffing (Costall et al., 1975), PE (Benassi-Benelli et al., 1979), and alterations in locomotor activity (Di Chiara et al., 1976), the receptor(s) mediating these effects have remained elusive. While the biphasic nature of many of these behavioral effects (i.e., yawning, PE, and locomotor activity) suggests that multiple receptors are involved, early hypotheses often attributed these effects to pre- and post-synaptic D_2 receptors (e.g., Mogilnicka and Klimek, 1977; Yamada and Furukawa, 1980; Urba-Holmgren et al., 1982; Dourish et al., 1985). A more detailed analysis of the temporal relation of these behaviors to other autoreceptor effects, such as decreases in extracellular dopamine, combined with pharmacologic studies aimed at manipulating synaptic dopamine levels, argues against the autoreceptor hypothesis, and has led to newer hypotheses attributing many of these effects to postsynaptic receptors of the D_2-like receptor family (e.g., Stahle and Ungerstedt, 1989b; Stahle and Ungerstedt, 1989a; Stahle and Ungerstedt, 1990; Stahle, 1992; Levant, 1997). The discovery of other D_2-like receptors, namely the D_3 (Sokoloff et al., 1990) and D_4 (Van Tol et al., 1991) receptors, together with the identification of agonists and antagonist displaying higher degrees of selectivity for the D_3 and/or D_2 receptors has allowed for the refinement of these hypotheses to incorporate specific roles for the D_2 and D_3 receptor in the receptor mediation of D_2-like behavioral effects.

One of the earliest hypotheses focused on a role for the D_3 receptor in the inhibition of locomotor activity, while the stimulation of locomotor activity observed at higher doses of D_2-like agonists was thought to be mediated by the D_2 receptor. This hypothesis was based on the findings that D_3-preferring agonists inhibited locomotor activity over low doses (Svensson et al., 1994) while D_3-preferring antagonists stimulated spontaneous locomotor activity when given alone (Waters et al., 1993). While this hypothesis has remained popular, validation has been complicated for several reasons. Not only have environmental and experimental conditions been shown to influence the locomotor effects of D_2-like agonists, (Szumlinski et al., 1997; Van Hartesveldt, 1997; Pritchard et al., 2003), but D_2 and D_3 antagonists often affect spontaneous locomotor activity when given alone (Waters et al., 1993; Sautel et al., 1995b; Millan et al., 2000) making the interpretation of their effects difficult. For example, while pharmacologic evidence for a role of the D_2 receptor in the stimulation of locomotor activity was provided in a recent study, Millan and colleagues (2004) were unable to confirm a role for the D_3 receptor in the locomotor inhibitory effects of D_2-like agonists, raising question about generality and reliability of this putative D_3-mediated behavioral effect. This hypothesis has been further complicated by the use of D_2 and/or D_3 receptor-deficient mice in the evaluation of the roles of the D_2 and D_3 receptors in the regulation of locomotor activity. Although the fact that D_3 receptor-deficient mice typically show increased levels of spontaneous locomotor activity (Accili et al., 1996; Xu et al., 1997) supports an inhibitory role of the D_3 receptor, D_2-like agonists typically have monophasic effects on locomotor activity in mice, with the inhibition of locomotor activity observed over a large range of doses (e.g., Pugsley et al., 1995; Pritchard et al., 2003) suggesting the involvement of a single receptor sub-type. Further support for the involvement of a single receptor sub-type is provided by the findings that D_3 receptor-deficient mice display a normal hypolocomotor response to D_2/D_3 agonists, while the hypolocomotor effects were absent in D_2 receptor-deficient mice (Boulay et al., 1999a; Boulay et al., 1999b), effects that are suggestive of a role for the D_2, but not D_3 receptor in the locomotor inhibitory effects of D_2-like agonists in mice. While this is contrary to popular hypotheses, these findings demonstrate the difficulty in evaluating and interpreting behavioral effects across different species.

Despite the apparent species differences with regard to the effects of D_2-like agonists on locomotor activity, the use of D_2 and D_3 receptor-deficient mice has been very useful in the characterization of other *in vivo* effects of D_2-like agonists. For example, based on the differential capacity of D_2-like agonists to induce hypothermia in D_2 and D_3 receptor-deficient mice it was hypothesized that the hypothermic effects of are mediated by their activity at the D_2, but not D_3 receptor, a hypothesis that was later supported by pharmacologic studies in rats (Boulay et al., 1999a; Boulay et al., 1999b; Chaperon et al., 2003). Interestingly, some of the behavioral effects of D_2-like agonists correspond to the induction of hypothermia (e.g., sniffing, and stimulation of locomotor activity), while others are often observed at lower doses (e.g., yawning, PE, and inhibition of locomotor activity). Based on this relation, and the biphasic nature of D_2-like agonist-induced yawning we hypothesized that the induction of yawning behavior by D_2-like agonists was mediated by their selective activation of the D_3 receptor, while the inhibition of yawning behavior at higher doses resulted from a concomitant activation of the D_2 receptor. The results of the studies reported herein provide strong support for this general hypothesis, and have extended it in several ways. In addition to providing pharmacologic validation for a specific role for the D_3 receptor in the induction of yawning behavior, the use of yawning as a D_3-mediated behavioral effect has provide the opportunity for determinations of *in vivo* D_3 and/or D_2 selectivity ratios to be made for D_2-like agonists and antagonists, as well as the identification of other elicited behaviors specifically mediated by the D_3 receptor.

Yawning as a D_3-mediated Behavior

In agreement with previous reports (e.g., Mogilnicka and Klimek, 1977; Urba-Holmgren et al., 1977; Yamada and Furukawa, 1980; Stancampiano et al., 1994), yawning was observed following administration of dopaminergic, cholinergic, and serotonergic agonists. Dose-dependent increases in yawning were observed following low doses of the D_2-like agonists, 7-OH-DPAT, apomorphine, bromocriptine, PD-128,907, pramipexole, quinelorane, and quinpirole, with the dose-dependent inhibition of yawning observed at higher doses resulting in a characteristic inverted U-shaped dose response curves for all agonists. While there were no differences in the effectiveness of the D_2-like agonists to induce yawning (with the exception of apomorphine, a non-selective D_1/D_2-like agonist), the rank-order potencies for the agonists to induce yawning were in general agreement with other D_2-like agonist-induced behavioral effects (e.g., Sanger et al., 1996) suggesting that yawning was mediated by the activation of a D_2-like receptor(s). Importantly, these studies are the first to provide strong pharmacologic support for a specific role of the D_3 receptor in the regulation of a D_2-like agonist-induced behavioral effect. D_3-preferring antagonists with varying degrees of D_3 selectivity (30-133 fold selective for the D_3 compared to D_2 receptor) produced a dose-dependent and selective inhibition of the induction of yawning behavior without altering the inhibition of yawning observed at higher doses. Conversely, a selective reversal of the inhibition of yawning observed at high doses of these agonists was observed following pretreatment with the D_2-preferring antagonist, L-741,626 (~10-fold selective for the D_2 compared to D_3 receptor), while no effect was observed on the induction of yawning. Together, these findings support the notion that the induction of yawning is mediated by a specific activation of the D_3 receptor, while the subsequent inhibition of yawning results from a concomitant activation of the D_2 receptor. In agreement with specific roles for the D_3 (induction) and D_2 (inhibition) receptors in the mediation of D_2-like agonist induced yawning, the non-selective D_2-like antagonist, haloperidol, produced rightward shifts of both the ascending and descending limbs of the dose-response curves for D_2-like agonist-induced yawning, while the D_1/D_5

antagonist, SCH23390, and the D_4-selective antagonist, L-745,870, did not alter the induction, or inhibition of PD-128,907-induced yawning.

Further evidence for a specific role of the D_3 receptor in the induction of yawning by D_2-like agonists was provided by the examination of the interactions of dopaminergic, cholinergic, and serotonergic systems in the regulation of yawning behavior. In agreement with previous reports (e.g., Holmgren and Urba-Holmgren, 1980; Yamada and Furukawa, 1980; Protais et al., 1995), and in support of common cholinergic pathway, scopolamine inhibited yawning induced by the indirect cholinergic agonist, physostigmine, the 5-HT_2 agonist, TFMPP, as well as the D_3-preferring agonist, PD-128,907. Conversely, the 5-HT_2 antagonist, mianserin, inhibited yawning induced by TFMPP, but not physostigmine or PD-128,907. A similar selectivity was observed with most of the D_3-preferring antagonists tested. PG01037, SB-277011A, and nafadotride dose-dependently inhibited the induction of yawning by PD-128,907 at doses that did not alter the induction of yawning by physostigmine or TFMPP, suggesting that their capacity to inhibit PD-128,907-induced yawning resulted from their antagonist activity at the D_3 receptor. Interestingly, while the moderately selective D_3 antagonist, U99194, preferentially inhibited PD-128,907-induced yawning, a suppression of yawning induced by PD-128,907, TFMPP, and physostigmine was observed at a dose of 10.0 mg/kg; an effect that is suggestive of a significant anti-cholinergic activity (Goudie et al., 2001). Taken together, the effects of D_2-like agonists alone and in combination with D_3- and D_2-preferring antagonists, along with the dopaminergic selectivity of the D_3 antagonists provide the strongest evidence to date in support of a specific role for the D_3 receptor in the regulation of a D_2-like agonist-induced behavior.

Yawning and Hypothermia: *in vivo* Selectivity of D_2-like Agonists and Antagonists

While the initial antagonist studies provide support for the hypothesis that yawning is differentially mediated by the D_3 (induction) and D_2 (inhibition) receptors, comparison of the relative potencies of D_2-like agonists and antagonists to affect the induction of yawning and hypothermia is important in validating the selectivity of the effect. To investigate this relationship, a series of D_2-like agonists with a wide range of selectivities for the D_2, D_3, and D_4 receptors were assessed for their capacity to induce yawning and hypothermia. Through this characterization, three distinct behavioral profiles emerged. D_3-preferring agonists (7-OH-DPAT, PD-128,907, pramipexole, quinelorane, and quinpirole) induced yawning over low doses with hypothermia occurring at higher doses that corresponded to the inhibition of yawning. Conversely, the D_2-preferring agonist, sumanirole, induced hypothermia at doses lower than those required to induce yawning, while D_4-preferring agonists did not induce either yawning or hypothermia at any dose tested. These differences in the relative potencies of D_3- and D_2-preferring agonists to induce yawning and hypothermia provide support for specific roles for the D_3 and D_2 receptors in the induction of yawning and hypothermia by D_2-like agonists, respectively. These notions were further supported by the differential effects of D_3- and D_2-selecitve antagonists on the induction of yawning and hypothermia by each of the D_2-like agonists. Pretreatment with a D_3-preferring antagonist resulted in an inhibition of yawning induced by the maximally effective dose for each agonist, while not affecting the low levels of yawning or induction of hypothermia observed at higher doses of these agonists. Conversely, the D_2-preferring antagonist, L-741,626, inhibited the induction of hypothermia, and reversed the inhibition of yawning resulting from high doses of the D_2-like agonists, while peak levels of yawning were unaffected. Taken together with the previous reports in rats (Chaperon et al., 2003; Collins et al., 2005) and mice (Boulay et al., 1999a; Boulay et al., 1999b), these findings not only provide strong pharmacologic support for specific roles for the D_3 receptor in the induction of yawning behavior, and D_2 receptor in the induction of hypothermia by D_2-like agonists, but also suggest that yawning and hypothermia may be useful for determinations of *in vivo* potency measures at the D_3 and D_2 receptors, respectively.

Although several of the agonists and antagonists assessed in these studies have been reported to be greater than 100-fold selective for the D_3 compared to D_2 receptors *in vitro*, the lack of a validated D_3-mediated behavioral effect has prevented similar determinations from being made *in* *vivo*. Thus, the relative potencies of D_2-like agonists to induce yawning and hypothermia were compared as a measure of *in vivo* D_2/D_3 selectivity, while similar determinations of *in vivo* D_2/D_3 selectivity were made for D_2-like antagonists based on comparisons of their relative potencies to inhibit D_2-like agonist-induced yawning and hypothermia. Of the agonists examined, pramipexole had the highest degree of D_3 selectivity (32-fold selective for the D_3 compared to the D_2 receptor), sumanirole had the highest degree of D_2 selectivity (10-fold selective for the D_2 compared to the D_3 receptor), while 7-OH-DPAT, PD-128,907, quinelorane, and quinpirole were all ~10-fold selectivity for the D_3 compared to D_2 receptor. While similar determinations of *in vivo* D_2/D_3 selectivity were possible for the D_2-preferring antagonist, L-741,626, the non-selective D_2-like antagonist, haloperidol, and the mildly preferential D_3 antagonist, nafadotride (all ~3-fold selective for the D_2 compared to the D_3 receptor), *in vivo* selectivity ratios could not be determined for the more selective D_3 antagonists, U99194, SB-277011A, and PG01037 due to the lack of effect on sumanirole-induced hypothermia. However, it should be noted that while the doses of the D_3-selecive antagonists were limited by solubility (PG01037 and SB-277011A) and anti-cholinergic activity (U99194), U99194 has been reported to inhibit the induction of hypothermia at a dose of ~13 mg/kg (Audinot et al., 1998), suggesting that similar determinations would have been possible if higher, presumably less selective doses could have been assessed. Regardless of these minor drawbacks, these findings suggest that assessing the effects of D_2-like agonists and antagonists on yawning and hypothermia may provide a valuable diagnostic tool in the characterization of *in vivo* D_3 and D_2 effects, respectfully.

D_2-like Agonist-Induced Yawning and Penile Erection

In addition to their capacity to induce yawning, D_2-like agonists are known to induce PE in a variety of species including mice, rats, monkeys, and man (Benassi-Benelli et al., 1979; Gisolfi et al., 1980; Lal et al., 1987; Rampin et al., 2003), however, the receptor(s) mediating this effect are still unknown. Recently, a specific role for the D_4 receptor in the induction of PE by D_2-like agonists has been suggested (Brioni et al., 2004; Melis et al., 2005; Enguehard-Gueiffier et al., 2006), however, other studies suggest that the pro-erectile effects of D_2-like agonists are mediated by D_2-like receptor(s) other than the D_4 receptor (Melis et al., 2006). In an attempt to determine the receptor(s) involved in the regulation of D_2-like agonist-induced PE, the potencies of a series of agonists with varying degrees of selectivities for the D_2, D_3, and D_4 receptors to induce PE were compared with their potencies to induce yawning, an effect that has previously been shown to be differentially mediated by the D_3 (induction) and D_2 (inhibition) receptors.

Similar to previous reports, D_3-preferring agonists induced significant levels of both yawning and PE over low doses, while both endpoints were inhibited at higher doses. However, unlike previous reports (Brioni et al., 2004;

Melis et al., 2005; Enguehard-Gueiffier et al., 2006), none of the D_4-selective agonists induced significant levels of PE or yawning. Importantly, the induction and inhibition of yawning and PE was observed over a similar range for all of the D_3-preferring agonists, suggesting that yawning and PE by D_2-like agonists are similarly mediated by the D_3 (induction) and D_2 (inhibition) receptors. This notion was further supported by the findings that a D_3-selective dose of PG01037 inhibited the induction of both yawning and PE by apomorphine and pramipexole, while a D_2-selective dose of L-741,626 reversed the inhibition of yawning and PE observed at higher doses of apomorphine and pramipexole. The D_4-selective antagonist, L-745,870, did not alter yawning or PE induced by apomorphine or pramipexole. These effects were confirmed by a dose-response analysis of the effects of a series of D_2-like antagonists with a range of selectivities for the D_2, D_3, and D_4 receptors on the induction of yawning and PE by the maximally effective dose of pramipexole. Yawning and PE were inhibited by roughly equivalent doses of D_3-selective, D_2/D_3, $D_2/D_3/D_4$, and D_2-preferring antagonists, while the D_4-selective antagonists, L-745,870 or Ro 61-6270 did not affect either yawning or PE. Taken together, the effects of the agonists alone and in combination with antagonists not only confirm the differential roles of the D_3 (induction) and D_2 (inhibition) receptors in the regulation of yawning, but also provide strong pharmacologic evidence to suggest that the induction of PE by D_2-like agonists is similarly mediated by an activation of the D_3 receptor, while the inhibition results from a concomitant activation of the D_2 receptor.

In summary, the experiments reported herein provide strong pharmacologic evidence supporting a specific role for the D_3 receptor in the induction of yawning by D_2-like agonists, while also supporting the notion that the inhibition of yawning observed at higher doses results from a concomitant activation of the D_2 receptor. Not only were D_3- and D_2-preferring antagonists found to differentially modulate the ascending, and descending limbs of the yawning dose-response curve, respectfully, but the inhibition of yawning observed at higher doses corresponded to the induction of hypothermia, a D_2-mediated effect that has been validated through both pharmacologic and genetic means. In addition, these studies strongly suggest that D_2-like agonist-induced yawning and PE are mediated by similar receptors, with the induction of PE resulting from an agonist activity at the D_3 receptor, and the inhibition of PE observed at higher doses from a concomitant activation of the D_2 receptor. Moreover, the identification of a behavioral effect specifically mediated by the D_3 receptor has allowed for determinations of *in vivo* D_2/D_3 selectivity to be made for both agonists and antagonists, and suggest that evaluation of D_2-like agonist-induced yawning, hypothermia, and PE will provide a valuable tool for the characterization of novel compounds with respect to agonist and antagonist activities at the D_2 and/or D_3 receptors.

Alternative Hypotheses and Potential Problems

Perhaps the most popular hypothesis regarding the receptors involved in the regulation of D_2-like agonist-induced yawning is the autoreceptor hypothesis which posits that the induction of yawning is mediated by presynaptic D_2 autoreceptors, while the subsequent inhibition of yawning results from the activation of postsynaptic D_2 receptors (e.g., Mogilnicka and Klimek, 1977; Yamada and Furukawa, 1980; Urba-Holmgren et al., 1982; Dourish et al., 1985). However, a considerable amount of evidence has been reported to support the notion that the induction and subsequent inhibition of D_2-like agonist induced yawning are both mediated by postsynaptic D_2-like receptors. For instance, not only do D_2-like agonists induce yawning with a shorter latency than the decreases in extracellular dopamine levels (Stahle and Ungerstedt, 1989a; Stahle and Ungerstedt, 1990), an effect mediated by presynaptic D_2-like autoreceptors (e.g., Di Chiara et al., 1976), but D_2-like agonist-induced yawning is unaffected by pretreatment with α-methyl-dl-p-tyrosine, but enhanced following a ~24 hr pretreatment with reserpine (Yamada and Furukawa, 1980; Arnt and Hyttel, 1984; Serra et al., 1986; Stahle and Ungerstedt, 1990; Fujikawa et al., 1996a). Together, these effects suggest that yawning is not affected by changes in extracellular dopamine levels, but the reserpine-induced enhancement suggests that yawning is affected by changes in the sensitizativity of postsynaptic D_2-like receptors. Finally, when considered with the finding that yawning is induced by (+)-3-PPP, a pre- and postsynaptic

D_2-like agonist, but not (-)-3-PPP (Stahle and Ungerstedt, 1984; Melis et al., 1989) a ligand that has been shown to act as a presynaptic D_2-like agonist, and a postsynaptic D_2-like antagonist (Hjorth et al., 1983; Koch et al., 1983), these studies provide strong evidence that the induction of yawning is mediated by an activation of postsynaptic D_2-like receptors.

Although new hypotheses regarding the regulation of D_2-like agonist-induced behaviors began to be formed with the discovery of the D3 receptor, and the development of D_3-preferring agonists, such as 7-OH-DPAT and PD-128,907 (e.g., D3-mediated inhibition of locomotor activity; Waters et al., 1993; Svensson et al., 1994), the induction of yawning behavior is still commonly thought of as a D_2 receptor-mediated behavior (e.g., Millan et al., 2000; Eguibar et al., 2003; Brown et al., 2006; Millan et al., 2008). However, many of these claims are based on findings reported before the identification of the D_3 receptor, or the effects of agonists and antagonists with limited selectivity (Morelli et al., 1986; Melis et al., 1987; Cooper et al., 1989; Stahle, 1992). Millan and colleagues have argued against a specific role for the D_3 receptor in the induction of yawning behavior based on the inability of purported D_3-selective antagonists to inhibit yawning at doses lower than those required to inhibit the induction of hypothermia, an effect they claim to be mediated by both D_2 and D_3 receptor activation (Millan et al., 2000; Millan et al., 2008). Moreover, they claim that the relatively high doses of SB-277011A and

PG01037 required to fully inhibit yawning (Collins et al., 2005; Collins et al., 2007) are excessively high and likely acting at both D_3 and D_2 receptors.

While it is true that relatively high doses of D_3-selective antagonists were required to fully inhibit the induction of yawning, significant decreases in yawning have been observed at doses of 10.0 mg/kg for both PG01037 and SB-277011A, dosed that are only slightly higher than those required to affect 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). The D_3 selectivity of PG01037 and SB-277011A is further supported by the fact that neither PG01037 nor SB-277011A affected the induction of hypothermia by either the D_2-preferring agonist, sumanirole (Collins et al., 2007), or the D_3-preferring agonist, 7-OH-DPAT (Ootsuka et al., 2007) at doses up to 56.0 mg/kg. These findings suggesting that, even at these relatively high doses, the effects of PG01037 and SB-277011A on yawning and PE are mediated by their antagonist activity at the D_3, and not D_2 receptor. Regardless of the selectivity of these effects, the relatively high doses of D_3-selective antagonists required to produce effects remain problematic due to the low nM affinities of these antagonists. Interestingly, a recent pharmacologic magnetic resonance imaging (phMRI) study has shown selective increases in regional cerebral blood volume (rCBV) within the NAcc shell compared to NAcc core (Grundt et al., 2007), brain regions with high and low levels of D_3 receptor expression, respectively (Diaz et al., 1995; Diaz et al., 2000; Stanwood et al., 2000a), following intravenous administration of low doses of PG01037 (1.0-2.0 mg/kg), suggesting that differences in the route of administration may significantly affect the potency of these antagonists.

As discussed earlier, the use of receptor-deficient mice has become a popular and powerful tool for the characterization of the involvement of specific receptors in a variety of diseases, as well as roles for specific receptor(s) in the regulation of a behavior. Unfortunately, receptor-deficient mice can not be used to validate the results of the pharmacologic characterization of the receptor regulation of D_2-like agonist-induced yawning, as unlike other species, mice do not yawn in response to D_2-like agonists (Li et al., in preparation). Regardless of this species difference, it is important to note that the induction of yawning and PE by D_2-like agonists has also been reported in monkeys (Pomerantz, 1991), and humans (Lal et al., 1989), suggesting that the analysis of D_2-like agonist-induced yawning and PE may prove to be useful in the evaluation of D_3 and D_2 receptor function and/or sensitivity in humans.

Implications for Human Disease

Since its discovery (Sokoloff et al., 1990), the D_3 receptor has received considerable interest as a pharmacologic target for the treatment of a variety of diseases including Parkinson's disease, depression, schizophrenia, restless leg syndrome and a variety of aspects of drug abuse (Joyce, 2001; Heidbreder et al., 2005; Newman et al., 2005; Clemens et al., 2006). While several D_2-like agonists, partial agonists, and antagonists are currently approved for use in humans (i.e., haloperidol, pramipexole, ropinirole, aripiprazole, and rotigotine) the receptor(s) mediating their therapeutic effects remain unknown. Elicited behavioral effects have proven useful for characterizing the effects of novel pharmacologic compounds with diverse mechanisms of action. However, despite the potential therapeutic utility of D_2- and/or D_3-selective ligands, relatively few agonists and/or antagonists highly selective for D_2 and/or D_3 receptor have been identified, making the determination of the receptor(s) mediating the behavioral and/or therapeutic effects of D_2-like agonists and antagonists difficult. These studies provide strong pharmacologic evidence for a specific role of the D_3 receptor in the induction of yawning and PE by D_2-like agonists, and suggest that they may provide a useful method for the determination of agonist and/or antagonist activities at the D_3 and D_2 receptors in a variety of species.

Interestingly, changes in normal levels of yawning have been a frequently observed, but often overlooked side-effect of treatment, or symptom of a variety of disease states including Parkinson's disease, depression, Huntington's disease, ALS, schizophrenia, and migraine (e.g., Daquin et al., 2001). While the presentation of yawning in patients does not necessarily represent dopaminergic activity (e.g., yawning induced by high doses of SSRIs; Beale and Murphree, 2000), several studies suggest that a more careful analysis of yawning behavior may be a useful diagnostic tool in the diagnosis and/or treatment of a variety of disease states. For instance, while several groups have used apomorphine-induced improvements in motor performance as a predictor of Parkinson's patients' sensitivity and responsiveness to dopaminergic therapeutics (e.g., Barker et al., 1989; Hughes et al., 1990; Gasser et al., 1992; Bonuccelli et al., 1993), only one of these studies also quantified the induction of yawning. In this study, increases in yawning were observed at doses that were roughly equivalent to those that produced motor improvements, but lower than doses that induced other "side-effects" such as, nausea, vomiting, and hiccups (Bonuccelli et al., 1993). Similarly, doses of pramipexole that have been shown to induce rotation and improve functional hand movements in hemi-parkinsonian monkeys, (Domino et al., 1997; Domino et al., 1998), correspond to doses that induce yawning in un-treated monkeys (unpublished data). Moreover, Parkinson's patients being treated with L-DOPA or apomorphine have reported increases in yawning just prior to the "on-state" transition (Goren and Friedman, 1998; O'Sullivan and Hughes, 1998) suggesting that D_3 receptor activation may play an important role in the anti-parkinsonian effects of a variety of dopaminergic therapeutics.

While L-DOPA remains the "gold-standard" for the initial treatment of Parkinson's disease (e.g., Weiner, 1999; Hely et al., 2000; Zesiewicz et al., 2007), the long-term use of L-DOPA is known to result in the development of dyskineasias, on-off motor fluctuations and tolerance, often requiring adjunctive therapies, or increases in dose and/or frequency of L-DOPA (e.g., Fabbrini et al., 2007; Jankovic and Stacy, 2007). However, the fact that newer, direct acting D_3-preferring agonists such as pramipexole, ropinirole, and rotigotine are generally equally effective at treating the symptoms of Parkinson's disease while reducing the risk of developing motor complications (e.g., Montastruc et al., 1999; ParkinsonStudyGroup, 2000; Inzelberg et al., 2003; Jenner, 2003; Marras et al., 2004; Hauser et al., 2007) has led many to rethink initiating therapy with L-DOPA. For instance, initiating therapy with pramipexole has been shown to effectively reverse the symptoms of Parkinson's disease while also reducing the occurrence of on-off motor fluctuations and slowing the onset of dyskinesias as compared to patients treated with L-DOPA alone (ParkinsonStudyGroup, 2000; Marek et al., 2002; Barone, 2003; Reichmann et al., 2006). Additionally, recent studies in laboratory animals (Jenner, 2003; Van Kampen et al., 2004; Iravani et al., 2006) and humans (ParkinsonStudyGroup, 2000; Clarke and Guttman, 2002; Izumi et al., 2007; Joyce and Millan, 2007) suggest that D_3-preferring agonists, such as pramipexole, may actually promote neurogenesis, raising the possibility that treatment with D_3-preferring agonists such as pramipexole and ropinirole may slow, or even reverse, the progression of Parkinson's disease. However, it should be noted that while patients treated with pramipexole and ropinirole have been shown to have a reduced risk of developing motor complications, recent studies have reported an increased risk of developing psychiatric and behavioral side-effects such as, hallucination, compulsive gambling, eating, shopping, and hypersexuality (Driver-Dunckley et al., 2003; Dodd et al., 2005; Nirenberg and Waters, 2006; Weintraub et al., 2006; Driver-Dunckley et al., 2007).

While the mechanism(s) responsible for the development of compulsive behaviors are currently unknown, it is thought to result from the prolonged stimulation of D_2 and/or D_3 receptors within the NAcc, or even a more general increase in the activity of the mesolimbic dopaminergic pathway (Dodd et al., 2005; Driver-Dunckley et al., 2007). Interestingly, repeated administration of relatively high doses of pramipexole (0.3-1.0 mg/kg twice daily) increase the expression of D_3 receptors within the NAcc shell, while repeated dosing with similarly high doses of quinpirole (1.0 mg/kg/day) have differential effects on the expression of D_3 (increase) and D_2 (decrease) receptors (Bordet et al., 1997; Maj et al., 2000; Stanwood et al., 2000b). Furthermore, similar patterns of quinpirole administration have been shown to induce a variety of compulsive-like behaviors in rats, including path stereotypies, checking behavior, and excessive responding for water in the presence of freely available water (Szechtman et al., 1998; Cioli et al., 2000; Amato et al., 2006; Dvorkin et al., 2006) suggesting these effects may result from changes in the relative expression levels of D_2 and D_3 receptors.

Similar changes in the relative expression levels of D_2 (decreased expression) and D_3 (increased expression) receptors have been reported in rats, monkeys, and humans following exposure to a wide variety of drugs of abuse including cocaine, ethanol and heroin (Segal et al., 1997; Le Foll et al., 2003; Spangler et al., 2003; Neisewander et al., 2004; Nader et al., 2006; Volkow et al., 2007). These decreases in D_2 receptor expression have been suggested to enhance the subjective and reinforcing effects of psychostimulants (Volkow et al., 1999; Morgan et al., 2002). The D_3 receptor is also thought to be important for a variety of aspects of reinforcement, including the reinforcing effects of stimuli associated with reward (Wolterink et al., 1993; Pilla et al., 1999; Gal and Gyertyan, 2006; Cervo et al., 2007; Collins and Woods, 2007). Thus, it is possible that the combined effects of increased D_3 and decreased D_2 receptor expression observed following prolonged exposure to drugs of abuse, L-DOPA, and D3-preferring agonists, such as pramipexole, may underlie the development of compulsive behaviors and/or addictive disorders similar to those observed in Parkinson's and restless leg patients.

Future Directions

When taken together the results of the studies described in this thesis provide strong evidence for specific roles for the D_3 (induction) and D_2 (inhibition) receptors in the regulation of D_2-like agonist-induced yawning and PE, while also confirming a specific role for the D_2 receptor in the mediation of D_2-like agonist-induced hypothermia. However, the fact that relatively high doses of D_3-selective antagonists are required to inhibit these yawning and PE, the lack of a highly selective D_2 antagonist, and the inability to validate these effects in receptor deficient mice are problematic. The following experiments are proposed to address these issues, and extend the use of D_2-like agonist-induced yawning and PE to gain insight into the effects of environmental and pharmacologic manipulations on the function of D_3 and D_2 receptors.

An important first step is to address the concerns of the selectivity of the effects of the D_3-selecitve antagonists, SB-277011A and PG01037, on yawning. The fact that relatively high doses of PG01037 and SB-277011A were required to inhibit the induction of yawning even though they possess the low nM affinities for the D_3 receptor has led some to question whether these effects are truly mediated by the D_3 receptor (Millan et al., 2008). However, the lack of effect on sumanirole-induced hypothermia, combined with the fact that increases in rCBV were observed following i.v. administration of low doses of PG01037 (Grundt et al., 2007) suggests that this may be due, at least in part, to poor pharmacokinetic properties following s.c. administration. To address this issue, it would be interesting to compare the potencies of these antagonists to inhibit the induction of yawning, PE, and hypothermia following administration by various routes of administration (s.c., i.p., and i.v.). These studies would not only provide valuable pharmacokinetic information, but given the relatively low solubility limits of SB-277011A and PG01037 (32.0 mg/ml) these studies may also allow for smaller doses to be used, thus increasing the probability of observing D_2 antagonist effects at higher doses. This would not only allow for *in vivo* D_3 selectivity ratios to be determined, but would also provide further evidence for the differential roles of the D_2 and D_3 receptors in the regulation of D_2-like agonist-induced yawning, PE, and hypothermia.

Although the limited selectivity of the currently available D_2-selective antagonist (L-741,626) was sufficient to make distinctions regarding specific roles for the D_2 versus D_3 receptor, the relatively low degree of *in vivo* D_2 selectivity (~3.2-fold) limits the information that can be gained through its use. For instance, although determinations of *in vivo* D_3 and/or D_2 selectivity are possible based on the relative potencies of D_2-like agonists to induce yawning and hypothermia, similar comparisons of *in vivo* effectiveness cannot be made. While this is in large part due to the fact that D_2 activity inhibits both of the behavioral endpoints identified as D_3-mediated, the limited selectivity of the agonists is also to blame. This is evident by the fact that pretreatment with L-741,626 resulted in increases in the maximal number of yawns and PE observed for all of the D_3-preferring agonists, including pramipexole. However, if it were possible to completely remove the inhibitory effects of the D_2 receptor, either with a more selective D_2 antagonists, or the use of small interfering RNA (siRNA) aimed at inhibiting the expression of the D_2 receptor it could allow for the emergence of monophasic dose-response curves for D_2-like agonist-induced yawning and PE, and the ability to compare D_3-preferring agonists with respect to their effectiveness at the D_3 receptor.

Alternatively, similar determinations of *in vivo* effectiveness may be possible in mice. Interestingly, although mice do not yawn in response to D_2-like agonists (Li et al., in preparation), D_2-like agonists have been shown to induce PE in mice (Rampin et al., 2003). Thus, it would be interesting to assess the capacity of D_2-like agonists to induce PE in wild-type, D_2, D_3, and D_4 receptor-deficient mice. This would not only allow for a genetic validation of the role of the differential roles of the D_3 (induction) and D_2 (inhibition) receptors, as proposed by the results of the pharmacologic studies reported herein, allow for determinations of *in vivo* selectivity to be made in mice, but may also allow for *in vivo* comparisons with regard to effectiveness at the D_3 receptor to be made. Moreover, the ability to evaluate D_2-like agonists and antagonists in mice is advantageous for several reasons including the ability to exploit various knock-out and knock-in mice to gain insight into potential differences with respect to the signaling pathways activated following D_2 and D_3 receptor activation.

Besides its obvious utility as a means to evaluate novel compounds for potential D_3 and/or D_2 agonist, partial agonist, or antagonist activity (e.g., Chen et al., in preparation), perhaps the most exciting use for D_2-like agonist-induced yawning is in the characterization of the effects of environmental and/or pharmacologic manipulations on the normal function of D_2 and/or D_3 receptors. For instance, we have recently exploited the differential roles of the D_3 (induction) and D_2 (inhibition) receptors in D_2-like agonist-induced yawning, PE, and hypothermia to assess the effects of food restriction on the function and/or sensitivity of the D_2 and D_3 receptors (Collins et al., 2008). While these studies were able to confirm previous reports that food restriction increases the sensitivity and/or function of the D_2 receptor (e.g., Carr et al., 2003), by assessing the effects of food restriction on pramipexole-induced yawning and PE, two D_3-mediated behaviors, it was possible to demonstrate that food restriction did not alter the sensitivity and/or function of the D_3 receptor. While this study focused on dietary manipulations of dopaminergic systems, similar studies could provide valuable information regarding how pharmacologic histories or disease states affect D_2 and/or D_3 function. In fact, studies in human suggest that heroin addicts have an enhanced yawning response to apomorphine as compared to controls (Casas et al., 1995; Guardia et al., 2002), suggesting that it would be possible to determine drug-induced changes in receptor expression might result in changes of yawning dose-response curves. Together, the changes in D_2-like agonist-induced yawning observed in food restricted rats, and human drug abusers suggest the analysis of D_2-like agonist-induced yawning may provide a valuable tool to elucidate the changes in D_2 and/or D_3 receptor sensitivity that may underlie other conditions such as the development of dyskinesias or compulsive behaviors following prolonged exposure to dopaminergic therapeutics such as pramipexole.

In conclusion, these studies are the first to provide strong pharmacologic evidence in support of behaviors specifically mediated by the D_3 receptor.

These findings have wide ranging implications for our understanding of agonists and antagonists acting at D_2 and D_3 receptors, as well as the involvement of the D_2 and D_3 receptors in the regulation of behavior. Additionally, D_2-like agonist-induced yawning and PE not only provides a method for the characterization of the functional selectivity of D_2-like agonists and antagonists in the whole animal, but will aid in the identification of novel compounds with agonist, partial agonist, or antagonist activities at the D_3 and/or D_2 receptors. Furthermore, D_2-like agonist induced yawning and PE will allow for an inexpensive and non-invasive method for the determining the effects of environmental and pharmacologic manipulations, as well as animal models of disease affect on function and/or sensitivity of D_2 and D_3 receptor.

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