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Dopamine D3 Regulation of Sensorimotor Gating

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Neurosciences

by

Wei-li Chang

Committee in charge:

> Professor Neal R. Swerdlow, Chair
> Professor Joan Heller Brown
> Professor Mark A. Geyer
> Professor George Koob
> Professor Gregory Light

2011

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Chair

University of California, San Diego

2011

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LIST OF ABBREVIATIONS

APO = apomorphine

cAMP = 3′-5′-cyclic adenosine mono-phosphate

CREB = cAMP response element-binding

D3R/DRD3 = dopamine D3 receptor

DA = dopamine

ERK1/2 = extracellular-signal-regulated kinase 1 or 2

GABA = γ-aminobutyric acid

GPCR = G protein-coupled receptor

GTPγS = guanosine-5'-O-(thiotriphosphate)

ICD = impulse control disorders

ICj = Islands of Calleja

KO = knockout

mCPu = medial caudo-putamen

NAc/NAC = nucleus accumbens

NAcC = nucleus accumbens core

NAcS = nucleus accumbens shell

OD = optical density

pCREB = phosphorylated cAMP response element binding

PD = Parkinson's Disease

PKA = protein kinase A

PKI = PKA inhibitor

PPI   = prepulse inhibition

PPX/PRA   = pramipexole

PS   = posterior striatum

RLS   = restless leg syndrome

ROP   = ropinirole

rt-PCR   = reverse transcription polymerase chain reaction

shRNA   = short hairpin RNA

SUM   = sumanirole

WM   = working memory

WT   = wild-type

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All chapters contained in this dissertation, in full or in part, have been published, submitted for publication, presented at professional conferences. The co-authors listed in publications and in other materials herein either assisted, directed, or supervised the research which forms the basis of this dissertation, or contributed to the conceptual development of the experiments described. The contributions of the dissertation author to each individual publication are described in the Appendix. The author also acknowledges the assistance of Ms. Nancy Callahan in preparation of this document. The citations for each chapter are listed below.

Chapter 6: Weber M, Chang WL, Breier M, Ko D, Swerdlow NR. (2008) Heritable strain differences in sensitivity to the startle gating-disruptive effects of D2 but not D3 receptor stimulation. *Behav Pharmacol* 19(8): 786-795.

Chapter 7: Weber M, Chang WL, Breier MR, Yang A, Millan MJ, Swerdlow NR. (2010) The effects of sumanirole on prepulse inhibition in rats. *Eur Neuropsychopharmacol* 20(6):421-5.

Chapter 8: Chang WL, Geyer MA, Buell MR, Weber M, Swerdlow NR. (2010) The effects of pramipexole on prepulse inhibition and locomotor activity in C57BL/6J mice. *Behavioural Pharmacology* 21(2):135-43.

Chapter 9: Chang WL, Richtand NM, Ahlbrand R, Swerdlow NR. The effects of pramipexole on locomotor activity in D3 mutant mice. Submitted for publication; Society for Neuroscience, Washington, DC, November 12-16, 2011, Submitted for presentation.

Chapter 10: Chang WL, Breier MR, Yang A, Swerdlow NR. (2011) Disparate effects of pramipexole on locomotor activity and sensorimotor gating in Sprague Dawley rats. *Pharmacol Biochem Behav*, in press.

Chapter 11: Chang WL, Zhou X, Schaukowitch K, Breier MR, Saint Marie RL, Swerdlow NR. Lentiviral vector-mediated suppression of dopamine D3 receptor expression in adult rats. Society for Neuroscience, San Diego, California, November 13-17,2010, poster # 767.15/Y7 .

and: Chang WL, Zhou X, Schaukowitch K, Breier MR, Saint Marie RL, Swerdlow NR. In Vivo Suppression of Dopamine D3 Receptor Expression in Adult Rats Using Lentiviral Vectors. American College of Neuropsychopharmacology meeting, Miami Beach, Florida, December 5-9, 2010.

Chapter 12: Chang WL, Saint Marie RL, Miller EJ, Breier MR, Weber M, Swerdlow NR. Effects of pramipexole on sensorimotor gating and cAMP/PKA signaling in the nucleus accumbens. Society of Biological Psychiatry, New Orleans, Louisiana, May 20-22, 2010.

and: Chang WL, Saint Marie RL, Breier MR, Yang A, Mizera M, Hammer RP, Swerdlow NR. Signaling pathways of the D3 agonist pramipexole in the rat ventral forebrain. Society for Neuroscience, Washington, DC, November 12-16, 2011, Submitted for presentation.

The following individuals, listed alphabetically, were co-authors of aforementioned work, and their names are listed under the titles of the chapters they contributed to.

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PUBLICATIONS

Papers:

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12. **Chang WL**, Weber M, Breier MR, Saint Marie RL, Swerdlow NR. Stereochemical, neuroanatomical, and receptor subtype selectivity of pramipexole effects on sensorimotor gating. *Submitted*

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ABSTRACT OF THE DISSERTATION

Dopamine D3 Regulation of Sensorimotor Gating

by

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Doctor of Philosophy in Neurosciences

University of California, San Diego, 2011

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The dopamine D3 receptor (D3R) has been implicated as a potential therapeutic target for several neuropsychiatric disorders. For example, schizophrenia is currently treated with medications that nonspecifically target D3 as well as D2 receptors. However, due to the homology between these receptor subtypes, the tools available for specifically studying the D3 system *in vivo* are limited. While intracellular signaling pathways of D3 receptors have been described using cell lines *in vitro*, the applicability of these models to intact neural systems and behavior remain unclear. This dissertation utilizes behavioral, pharmacological, genetic, and cellular/molecular techniques in mice, rats, and humans to

examine the role of D3 receptors in regulating sensorimotor gating. Sensorimotor gating is the inhibition of a motor response by a sensory event and can be operationally measured with prepulse inhibition (PPI) of acoustic startle. PPI - the reduction in response to a startling stimulus when it is preceded by a weak prestimulus - is diminished in patients with schizophrenia and these deficits may be related to dopaminergic dysfunction. In animal models, PPI is disrupted acutely by dopaminergic agonists, and blockade of this effect predicts clinical efficacy of antipsychotics. After determining that the D3-preferential agonist pramipexole offers advantages for studying the PPI-disruptive effects of D3 stimulation, studies in this thesis described the stereochemical, anatomical and receptor-specific effects of this drug on PPI, and dissociated these effects from those on other behaviors. Using conditions that simulated D3R-mediated PPI deficits, brain regions relevant to PPI circuitry were analyzed for corresponding intracellular signaling changes. Specific signaling elements were altered by both D3 and D2 stimulation, but Fos expression in the nucleus accumbens appeared to be differentially suppressed by pramipexole and not the selective D2 agonist, sumanirole, and consistent findings were detected using D2- and D3-preferential antagonists. Collectively, these studies establish a strategy for parsing the anatomical, neurochemical and molecular substrates underlying the regulation of sensorimotor gating by forebrain D2 vs. D3 receptors, and identify divergent mechanisms that might be important targets for future drug development.

INTRODUCTION:

Background and significance of D3 receptors and sensorimotor gating

A: Dopamine Receptor Subtypes:

Five different subtypes of dopamine (DA) receptors exist in mammals, and it has been over 30 years since these metabotropic, seven transmembrane domain, G protein-coupled receptors (GPCRs) were grouped into two classes based on their structural, pharmacological, and functional characteristics (Kebabian and Calne, 1979). The D1-like receptor class consists of both D1 and D5 receptor subtypes, while the D2-like receptor class is composed of the D2, D3, and D4 receptor subtypes. Human D3 receptors have 46% amino acid sequence homology with the D2 receptor, with 78% homology in the transmembrane-spanning domains; rat D2 and D3 receptor have 52% homology with 75% in transmembrane-spanning domains (Sokoloff et al., 1990). In addition to sequence homology, the spatial orientation of conserved amino acids is nearly identical between D2 and D3 receptors (Livingstone et al., 1992).

In vivo, alternate dopamine D2 receptor gene splicing results in a short (D2S) and a long (D2L) isoform. The D2L receptor contains 29 more amino acids in the third cytoplasmic loop as compared to D2S, and D2S has been associated with presynaptic functions while D2L has been associated with postsynaptic functions (Giros et al., 1996; Lindgren et al., 2003; Monsma et al., 1989; Usiello et al., 2000). D3 receptors also function both presynaptically and postsynaptically (Joseph et al., 2002; Missale et al.,

1998; Schwartz et al., 2000), though to date no alternate isoforms have been identified. Unless otherwise noted, the terms D2 and D2L are used interchangeably.

1. Anatomical Distribution: D3Rs are localized in mesolimbic and limbic regions, which may be of particular relevance to schizophrenia pathology and/or therapeutics; highest D3R densities are found in the nucleus accumbens (NAc) and the Islands of Calleja in rats and primates, including humans (Landwehrmeyer et al., 1993; Levesque et al., 1992; Sokoloff et al., 2006; Stanwood et al., 2000); lower densities of D3Rs are also found in the ventral pallidum, mediodorsal thalamus, cerebral cortex, ventral tegmental area, and amygdale (Bouthenet et al., 1991; Diaz et al., 1995). D3 receptor transcripts are located in mesencephalic areas rich in DA cell bodies (cf. Heidbreder et al., 2005; Sokoloff et al., 2006) and are coexpressed with D1 and D2 receptors in the NAc (Le Moine and Bloch, 1996).

Among DA receptors, the D3 subtypes have the highest affinity for endogenous DA (Levant 1997; Sokoloff et al., 2001). However, D3 receptors are expressed with roughly 10-fold lower density than D2 receptors (Sokoloff et al., 1990).

2. Signaling Pathways: D1-like receptors are mostly G_s-protein linked and enhance the production of 3′-5′-cyclic adenosine monophosphate (cAMP). Members of the D2-like receptor class are generally thought to function through inhibition of cAMP accumulation (De Camilli et al., 1979; Stoof et al., 1981), though a great deal is still

unknown about the intracellular effects of D2-like receptor activation. It has previously been shown that human DA D2L receptors can efficiently couple to all four $G\alpha_{i/o}$ subtypes ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{o1}$) (Leof , 2000), while D3 coupling to $G\alpha_{i/o}$ G-proteins has largely been inferred based on the ability of pertussis toxin to block [^{35}S]guanosine-5'-O-(thiotriphosphate) ([^{35}S]GTPγS) binding in D3 receptor-expressing cells. Some studies have suggested that D3 receptors selectively couple on to $G\alpha_{o1}$ (Beom et al., 2004), though even this may require the expression of adenylyl cyclase V (Zaworski et al., 1999). While all D2-like receptors were originally thought to act through inhibition of cAMP accumulation, inhibition mediated by the D3 receptor is often modest and may even be absent (Barrot et al., 2002; Chio et al., 1994; MacKenzie et al., 1994; Tang et al., 1994). G proteins linked to D3Rs may also activate the MAPK/ERK signaling pathway (Brami-Cherrier et al., 2009; Bruins Slot et al., 2007; Cussac et al., 1999). Alteration of PKA or MAPK/ERK activity by D3Rs can ultimately lead to changes in expression of immediate early genes such as *c-fos,* which act as rapidly inducible transcription factors that may mediate the effects of prolonged changes in DAergic activity (Morris et al., 2000).

Much of our current understanding of D3R signal transduction mechanisms is based on *in vitro* heterologous expression systems in cell lines. One limitation of this approach is that it relies on signaling elements present in the host cell or co-transfected into the cells, and thus the observed pathways might not be fully relevant to signaling that is normally found in the brain (Kenakin, 1996). A major goal of the studies outlined in this dissertation was to link changes in elements of the hypothesized D3 receptor

signaling cascade (Figure 0.1) to alterations of sensorimotor gating, a process explained in more detail below.

Figure 0.1: Hypothesized intracellular signaling cascade of D3Rs. D2-like receptor activation inhibits adenylate cyclase. For clarity, the pathway has been simplified; several steps and inter-relationships have been omitted. White arrows indicate multiple or uncharacterized steps in the pathway. Adapted from (Conn et al., 2005; Cussac et al., 1999; Kelly et al., 2007)
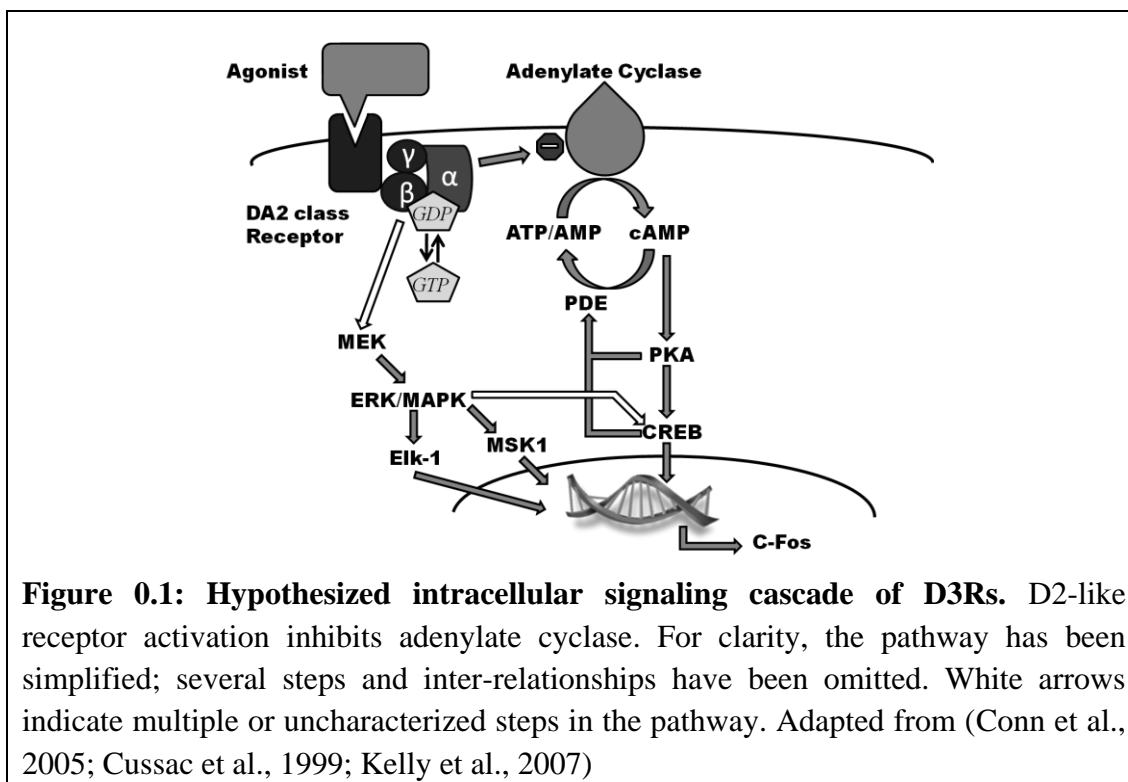
3. Clinical relevance: Many neuropsychiatric disorders, including the schizophrenias, appear to be characterized by disturbances of the dopaminergic (DAergic) systems (Carlsson, 1988; Carlsson and Carlsson, 2006; Davis et al., 1991; Randrup and Munkvad, 1972). Accordingly, all antipsychotic medications functionally antagonize activity at D2-like (D2, D3 and D4) DA receptor subtypes (Creese et al., 1996). All currently available antipsychotics bind non-specifically to antagonize both D2 and D3 receptors (Farde et al., 1989; Farde and Nordstrom, 1993; Sokoloff et al., 2006), and some of this non-specific activity may contribute to motoric and dysphoric side

effects that lead to low patient compliance and high discontinuation (Lieberman et al., 2005). Some adverse effects of antipsychotics appear to reflect reduced DA activity in regions within the D2R, but not D3R distribution. Conceivably, drugs that preferentially target D3Rs might provide anatomical selectivity, leading to clinical efficacy with fewer adverse side effects, as some preclinical data suggests (Joyce and Millan, 2005; Reavill et al., 2000). A preferential role of D3 receptors in the therapeutic vs. adverse actions of antipsychotics is also suggested by the observation that both adverse effects in humans (e.g. Parkinsonian symptoms and tardive dyskinesia) and D2 receptor mRNA and binding in rats fluctuate over the course of chronic antipsychotic exposure; in contrast, D3 mRNA expression and D3R binding during sustained antipsychotic administration remain unchanged in many brain areas (Fishburn et al., 1994; Gurevich et al., 1997; Schwartz et al., 2000; Tarazi et al., 1997).

Analyses of post-mortem brain tissue from patients with schizophrenia have revealed elevated levels of D3 receptor expression in patients who were drug-free compared to medicated at the time of death (Gurevich et al., 1997). Additionally, there have been reports of an association between homozygosity at the Ser9Gly D3 polymorphisms and schizophrenia (Crocq et al., 1992; Dubertret et al., 1998; Williams et al., 1998), though this association may only contribute very modestly to schizophrenia susceptibility (Jonsson et al., 2004).

The D3 receptor system has been implicated as a therapeutic target for Parkinson's Disease and depression as well, and evidence supporting these claims are reviewed elsewhere (cf. Sokoloff et al., 2006). The studies outlined in this dissertation

focused on animal models more relevant to antipsychotic development, and the potential for the role of D3 receptor activity in anti-Parkinsonian and antidepressant medication development was not explicitly explored.

B. Prepulse Inhibition of Startle:

The startle reflex is a constellation of defensive responses to a sudden, intense stimulus and is mediated by a primary mammalian acoustic startle circuit (Davis et al., 1982). This simple reflex demonstrates several conceptually important forms of plasticity, including "prepulse inhibition" (PPI), which is the normal suppression of startle when the intense startling stimulus is preceded by a weak prestimulus (Graham, 1975; Hoffman and Searle, 1968; Ison et al., 1973) (Fig. 0.2).

Figure 0.2: Prepulse inhibition (PPI) is a decrease in startle amplitude when the startling "pulse" is preceded by a weak "prepulse". The amount of PPI is expressed as: $100- (r/R \times 100)$
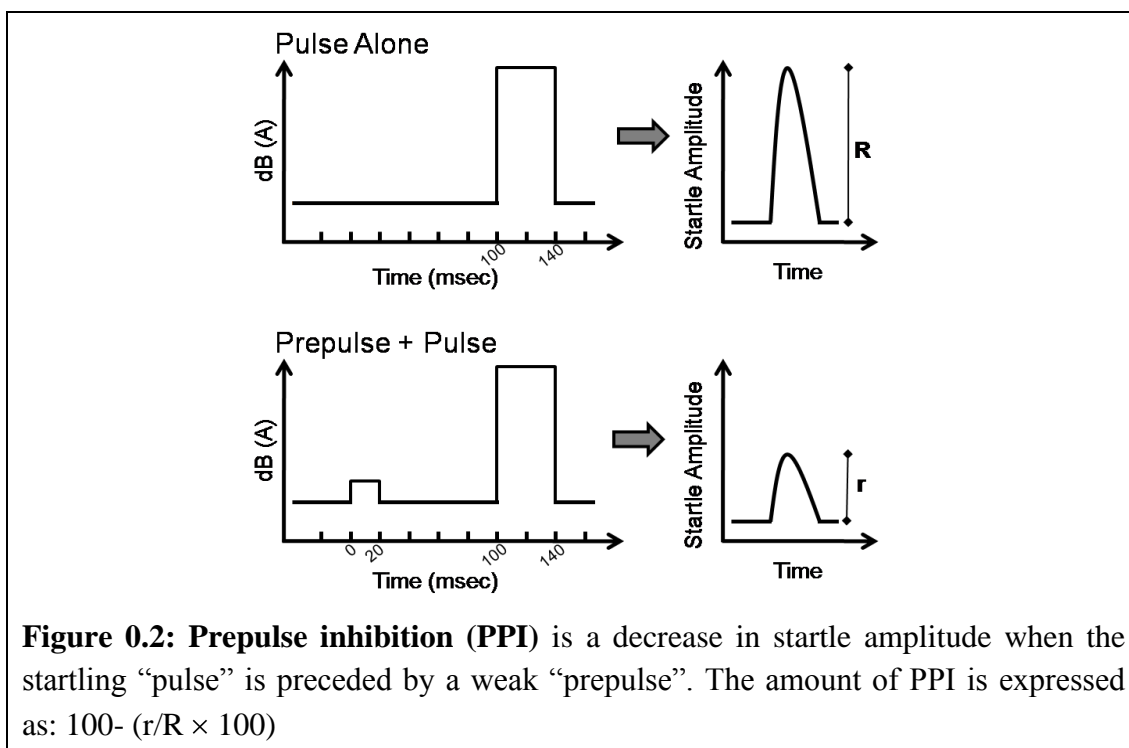
In one model of PPI, a weak sensory event (the prepulse) activates neural processes that blunt the motor response to subsequent stimuli during a brief temporal window (roughly from 10-300 ms in a rat (Swerdlow, 1993)). Conceptually, this diminished responsivity - termed "sensorimotor gating" - might reflect mechanisms that normally serve to protect information contained in the weak lead stimulus, so that it can be processed without interference from subsequent events. This "information protective" gating process might then contribute to the ability to segregate a continuous stream of sensory and cognitive information, and thereby selectively allocate attentional resources to salient stimuli. A substantial, pathological breakdown of this automatic gating mechanism has been proposed to impair the orderly, hierarchical processing of sensory events and result in flooding of sensory information with a fragmenting impact on cognition (McGhie and Chapman, 1961). Importantly, while this model has gained traction for its heuristic value in understanding the clinical implications of sensorimotor gating deficits, there is no direct, compelling evidence for a direct causal link between PPI deficits and neurocognitive disturbances related to "sensory flooding." Nonetheless, a substantial convergence of clinical and preclinical findings have fostered great interest in identifying its neural and molecular substrates of PPI, as a basis for understanding the pathophysiology and therapeutics of several brain disorders, including schizophrenia (cf. Swerdlow et al., 2008b).

1. Significance: PPI is significantly reduced in specific brain disorders, many of which share two features: 1) clinical evidence of impaired cognitive or sensorimotor

inhibition; and 2) dysfunction in limbic cortico-striato-pallido-thalamic (CSPT) circuits that regulate PPI. At least 10 research groups have reported PPI deficits in schizophrenia (Borrell et al., 2002; Braff and Freedman, 2002; Braff et al., 1978, 1992, 2001, 2005, 2007; Cadenhead et al., 2000; Hazlett et al., 1998; Kumari et al., 2007; Ludewig and Vollenweider, 2002; Oranje et al., 2002; Schal et al., 1996; Weike et al., 2000), making this finding among the most replicated quantitative markers of brain-based disturbances in this disorder (Braff and Freedman, 2002). There are also reports of deficient PPI in Huntington's Disease (Swerdlow et al., 1995; Valls-Sole et al., 2004), Tourette Syndrome (Castellanos et al., 1996; Swerdlow et al., 2001b), and Obsessive Compulsive Disorder (Schall et al., 1996; Swerdlow et al., 1993). PPI may be a particularly valuable model for studying the neural substrates of schizophrenia, because of the relevance of PPI "anatomy" to the pathophysiology of schizophrenia, and because deficits in gating of cognitive and sensory information are clinically important features of this disorder. Indeed, functional impairment in schizophrenia patients has been associated with low PPI levels (Swerdlow et al., 2006b). While we do not know the precise neural substrates of PPI deficits in schizophrenia, imaging and neuropathological studies have revealed abnormalities at several levels of the startle "gating circuitry" in schizophrenia patients, including the hippocampus, NAc, striatum, globus pallidus and thalamus (Akil et al., 1999; Bogerts, 1993; Bogerts et al., 1985, 1990; Harrison and Eastwood, 1998; Jakob and Beckmann, 1986; Kumari et al., 2007; McCarley et al., 1999; Pakkenberg, 1990; Silbersweig et al., 1995; Smith et al., 2003). Perhaps of most immediate relevance to this dissertation, PPI has been widely used in animal models to predict clinical efficacy of novel antipsychotic compounds. For example, the ability of antipsychotics to prevent the

PPI-disruptive effects of DA agonists correlates significantly (r = 0.99) with their clinical potency (cf. Swerdlow et al., 1994).

2. Pharmacology and Anatomy: PPI is regulated by cortical structures (mesial temporal and medial prefrontal cortex) and subcortical structures (basolateral amygdala, striatum, pallidum, and pontine tegmentum (cf. Koch and Schnitzler, 1997; Swerdlow et al., 2001a)). The neural circuitry of PPI is described in several published reviews (e.g. Swerdlow et al., 2000; 2001a; 2008b). The conclusions most relevant to the studies to be described in this thesis are that: 1) PPI is potently regulated by forebrain DA activity; 2) this DAergic regulation is mediated by D2-family receptor activation in forebrain regions, particularly the NAc, and 3) by the subsequent reduction in GABA release "downstream" in the VP and perhaps other NAc projection targets.

Models of PPI "circuitry" have previously left a mechanistic "black box" between the point of D2-family receptor activation, and that of reduced VP GABA release. However, it is now apparent that elements of NAc D2-like receptor G-protein signal transduction pathways mediate the impact of DA agonists on PPI. Stimulation of D2-family receptors disrupts PPI by activating inhibitory $G\alpha_{i/o}$ proteins in the NAc, thereby reducing cAMP signaling by inhibiting adenylate cyclase activity (Culm et al., 2004). This mechanism is sensitive to adaptation by increased protein kinase A (PKA) activity and CREB phosphorylation; blunted D2 effects on PPI can be initiated and sustained by viral-mediated NAc p-CREB overexpression (Krupin et al., 2007). NAc PKA activation

appears to blunt the ability of D2-like activation to disrupt PPI: the PPI-disruptive effects of quinpirole are blocked by activation of PKA via intra- NAc infusion of the cAMP analog, Sp-cAMPS (Culm et al., 2004). Antipsychotics increase striatal PKA activity and induce CREB phosphorylation (Dwivedi et al., 2002; Pozzi et al., 2003), and under specific conditions (e.g. low basal inhibitory drive (cf. Geyer et al., 2001; Swerdlow et al., 2006c)) can enhance PPI. Lastly, reduced PPI in postpartum rats is associated with reduced NAc cAMP content (Byrnes et al., 2007). Surely, this biology is not unique to PPI: NAc CREB phosphorylation appears to regulate behavioral responses to stimuli ranging from anxiogenic (Barrot et al., 2002) to rewarding (Carlezon et al., 1998).

While the DAergic regulation of PPI is primarily ascribed to D2-like receptors, there is some evidence that stimulation of D3Rs in rats leads to a disruption in PPI. Some preferential D3 agonists have been demonstrated to reduce PPI in rats, including ropinirole, 7-OH-DPAT, and PD128907 (Caine et al., 1995; Swerdlow et al., 1998a; Varty and Higgins, 1998; Zhang et al., 2007), but the mechanisms responsible for the D3-regulation of PPI are not yet understood. Drugs acting "downstream" from D2/D3 receptors, in D2/D3-linked signal cascades, might have more selective antipsychotic properties, with limited side effect liability (Bosier and Hermans, 2007; Mailman, 2007). By identifying sites of divergence in these pathways that differentially control the D2- vs. D3-regulation of PPI, it might be possible to choose strong inference-based targets for novel drugs. Ultimately, these novel drugs would be used to more effectively treat disorders characterized by defects in sensorimotor gating.

D. Aims of study:

The studies detailed in this dissertation aim to determine the behavioral and intracellular signaling effects of D3 receptor activation, linking the changes observed in awake behaving animals after D3 stimulation with changes in signaling molecules observed in specific forebrain regions. Effects of D3 receptor activation on behavior will first be characterized, and then methods for eliciting sensorimotor gating changes will be optimized and dissociated from other behavioral changes. Scientific studies in different species all have unique strengths and weakness; while rats are the primary animal model for this line of inquiry, studies in mice and humans are also included to identify areas of cross-species convergence and divergence in D3 receptor effects. Finally, anatomical and cellular/molecular substrates mediating sensorimotor gating effects of D3 receptor activity will be identified. In this manner, we will begin to translate information obtained from *in vitro* studies of D3 receptor effects into a model of the D3 receptor systems in the mammalian brain that regulate the clinically-relevant process of sensorimotor gating.

CHAPTER 1:

Pramipexole effects on startle gating in rats and normal men

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A. Abstract:

1. Background: Dopamine D3 receptors regulate sensorimotor gating in rats, as evidenced by changes in prepulse inhibition (PPI) of startle after acute administration of D3 agonists and antagonists. Here, we tested the effects of the D3-preferential agonist, pramipexole, on PPI in normal men and Sprague Dawley rats.

2. Methods: Acoustic startle and PPI were tested in clinically normal men, comparing the effects of placebo vs. 0.125 mg (n=20) or placebo vs. 0.1875 mg (n=20) pramipexole, in double blind, crossover designs. These measures were also tested in male Sprague Dawley rats using a parallel design (vehicle vs. 0.1 mg/kg (n=8), vehicle vs. 0.3 mg/kg (n=8) or vehicle vs. 1.0 mg/kg pramipexole (n=8)). Autonomic and subjective measures of pramipexole effects and several personality instruments were also measured in humans.

3. Results: Pramipexole increased drowsiness, and significantly increased PPI at 120 ms intervals in humans; the latter effect was not moderated by baseline PPI or personality scale scores. In rats, pramipexole causes a dose-dependent reduction in long-interval (120 ms) PPI, while low doses actually increased short-interval (10-20 ms) PPI.

Effects of pramipexole on PPI in rats were independent of baseline PPI and changes in startle magnitude.

4. Conclusions: The preferential D3 agonist pramipexole modifies PPI in humans and rats. Unlike indirect DA agonists and mixed D2/D3 agonists, pramipexole increases long interval PPI in humans, in a manner that is independent of baseline PPI and personality measures. These findings are consistent with preclinical evidence for differences in the D2- and D3-mediated regulation of sensorimotor gating.

B. Introduction:

Prepulse inhibition (PPI) is an operational measure of sensorimotor gating, in which a startle response to an intense stimulus is automatically suppressed by a weak lead stimulus (Graham 1975). In humans, PPI is typically assessed using electromyographic measures of the blink response, while in rodents, PPI is typically assessed by measuring whole body startle. PPI deficits are found in several neuropsychiatric disorders (Braff et al., 1978; cf. Braff et al., 2001; cf. Swerdlow et al., 2008b), and the biology of these deficits has been the focus of intense study in humans and animal models.

In rats, dopamine (DA) agonists generally reduce PPI, although these effects are highly strain-sensitive (Swerdlow et al., 2004a, 2004b). For example, the mixed D1/D2 agonist apomorphine (APO) disrupts PPI in albino Sprague Dawley rats but not in pigmented ACI rats, while in hooded Long Evans rats, APO increases PPI at short prepulse intervals and reduces it at long prepulse intervals (Swerdlow et al., 2004a,

2004b). In humans, the effects of DA agonists on PPI differ across groups distinguished by baseline PPI levels and/or personality dimensions. For example, Bitsios et al. (2005) reported PPI-disruptive effects of the DA agonists pergolide and amantadine only among men characterized by high baseline PPI levels, Giakoumaki et al. (2007) reported a reduction in PPI after administration of the mixed D2/D3 agonist ropinirole to healthy men whose baseline PPI was > 30%, and we reported similar baseline-dependent effects of amphetamine on PPI in healthy men (Swerdlow et al., 2003b) and women (Talledo et al., 2009). Furthermore, amphetamine has been reported to disrupt PPI only among normal subjects with high scores on measures of novelty seeking or sensation seeking (Hutchison et al., 1999; Talledo et al., 2009). In some subgroups of normal humans (e.g. those characterized by low baseline PPI or novelty seeking), DA agonists can actually increase PPI (Talledo et al., 2009). Some evidence suggests that differences in PPI sensitivity to DA agonists in rats and humans may be mediated in part by differences in brain regional activity of catechol-O-methyl transferase (COMT) (Shilling et al., 2008; Roussos et al., 2008b; Talledo et al., 2009).

The present study tested the effects of pramipexole, a non-ergot preferential D3 agonist, on PPI. While the vast majority of studies of the DAergic regulation of PPI in rats and humans focus on the role of D1 and D2 receptors, more recent evidence suggests that PPI in both species may also be regulated by D3 receptors. For example, Roussos et al. (2008a) reported that PPI differed significantly across humans characterized by Gly/Gly vs. Ser/Ser variants of the D3 receptor Ser9Gly polymorphism. Pramipexole has preferential affinity for D3 vs. D2 receptors, with an in-vitro D3:D2 preference of 7.8:1

(Piercey et al., 1996, Svensson et al., 1994a; cf. Kvernmo et al., 2006) relative to the high affinity state of the D2 receptor. Millan et al. (2002) determined the D3:D2 preference of pramipexole to be 90:1 relative to the short isoform of the human receptor (D2S), and 160:1 relative to the long isoform (D2L). In comparison, ropinirole is relatively less preferential for D3 receptors (e.g. in vitro D3:D2 preference = 1.3:1 (cf. Kvernmo et al., 2006), with preference of 18:1 relative to D2S and 25:1 relative to D2L (Millan et al., 2002)).

Our group reported that pramipexole disrupts PPI in laboratory rats, and that this effect is relatively insensitive to selective D2 blockade, but is opposed by functional D3 antagonists (Weber et al., 2008a, 2009b). In addition, strain differences in the PPI-disruptive effects of indirect DA agonists (amphetamine (Swerdlow et al., 2003a), mixed D1/D2 agonists (apomorphine (Swerdlow et al., 2001c)) and D2-preferential agonists (quinpirole (Swerdlow et al., 2001c)) are not detected with pramipexole (Weber et al., 2008a), suggesting that its PPI-disruptive effects are mediated differently from those of non-D3 DA agonists. Here, we examined the effects of pramipexole on PPI in normal men, also assessing the potential moderating impact of baseline PPI and personality scale scores.

C. Methods:

1. Human testing: The methods used in these studies were very similar to those used in studies described in recent reports (Swerdlow et al., 2003b), were approved by

the UCSD Human Subjects Institutional Review Board, and were approved and supported by the National Institute of Mental Health. Forty (40) R handed men (Table 1.1) completed testing; the study involved a phone contact and three laboratory visits. Phone screening procedures were identical to those described in previous reports from our group (Swerdlow et al., 2003b).

After passing a telephone interview, subjects came to the laboratory for a screening examination, during which the senior investigator (NRS) informed subjects of the potential risks and benefits of the study. Subjects read and signed a consent form for study participation, underwent a physical examination and electrocardiogram to rule out exclusionary medical conditions, a modified Structured Clinical Interview for DSM Disorders (non-patient edition; SCID) (First et al., 1997) to rule out Axis-I diagnoses, and completed a urine toxicology test with exclusion for any illicit drug. Audiometry confirmed hearing threshold < 40 dB(A) at 1000 Hz. Subjects also completed a limited test of the acoustic startle reflex to screen for a minimum eyeblink startle magnitude of 50 units (1.22 µV/unit) using 118 dB(A), 40 ms noise pulses.

Subjects completed the following questionnaires: 1) the Tridimensional Personality Questionnaire (TPQ) (Cloninger, 1987) to assess the relationship between novelty seeking scores (NS) and sensitivity to the effects of pramipexole on PPI, based on reports that high NS individuals are most sensitive to the PPI-disruptive effects of indirect DA agonists (Hutchison et al., 1999; Talledo et al., 2009) and the PPI-enhancing effects of antipsychotics (Swerdlow et al., 2006c); 2) the Sensation Seeking Scale (SSS) (Zuckerman et al., 1972), based on reported increased sensitivity to DA agonists in

individuals scoring high on this measure (Hutchison et al., 1999); and 3) the Eysenck Personality Questionnaire (EPQ) (Eysenck and Eysenck, 1994). Subjects who passed screening criteria were tested 6-8 d later, and retested 6 - 8 d after their first experimental session.

The study had a 2-test within-subject design for each dose of pramipexole. In other words, one group of subjects (n=20) was tested with placebo and 0.125 mg pramipexole, and a second group of subjects (n=20) was tested with placebo and 0.1875 mg pramipexole. On test days, subjects arrived at 0830, ate a standardized breakfast, and pramipexole (0.125 or 0.1875 mg) (Boehringer Ingelheim GmbH, Ingelheim, Germany) or placebo was administered at 0930. Startle testing began 60 min after pill administration. Heart rate and blood pressure were determined (sitting position, brachial cuff), and subjects completed a symptom rating scale every 30-45 min; the first one occurred before pill ingestion. Symptom-rating visual analog scales (VAS) were designed to assess general somatic and psychological symptoms and level of consciousness (modified from Bond and Lader 1974; Bunney et al., 1999; Norris, 1971). Subjects made a single, vertical mark representing their current state along on a 100 mm line (0 mm represents "not true" and 100 mm represents "true"). Ratings assessed several states: "happy", "queasy", "dizzy", "drowsy" and perceptual sensitivity. Details of these rating scales are found in Swerdlow et al., (2002), and included prompts such as "Normal sounds seem unusually intense or loud".

For startle testing, subjects sat upright and were directed to look straight ahead, and to stay awake. Two miniature Ag/AgCl electrodes were positioned below and to the

outer canthus of each eye over orbicularis oculi; ground electrode was positioned behind the L ear (R < 10 kΩ). EMG activity was band-pass filtered (1 − 1000 Hz) and 60 Hz notch filtered, digitized, and 250 1 ms readings were recorded starting at startle stimulus onset. Acoustic startle stimuli were delivered by Telephonics (TDH-39-P, Maico) headphones. A background 70 dB(A) white noise was continuous throughout the session. Test sessions began with a 3 min acclimation period; during this period, the number of spontaneous eyeblinks were counted by a remote observer using a RadioShack security camera system (model 49-2513) (inter-observer R=0.97). This was followed by 42 trials with 6 conditions repeated in pseudorandom order: a 118 dB(A) 40 ms noise burst alone (pulse alone), and the same 118 dB(A) 40 ms noise burst preceded 10, 20, 30, 60 or 120 ms by a prepulse (5 ms burst) 16 dB over background. A variable inter-trial interval averaged 20 s (15 − 25 s). The test session was structured identically to that described in our previous studies of DA agonist effects on PPI in humans (Swerdlow et al., 2003b; Talledo et al., 2009). On completion of this startle test, additional autonomic and subjective rating measurements were obtained, as were additional "pilot" psychophysiological measures, including a visual latent inhibition task. Data from these subsequent tests are not included in this analysis.

The primary reasons for disqualification were that subjects had low screening startle magnitude (n=22), withdrew from testing prior to the second test day (n=3) or had a SCID-based diagnosis (n=14) (First et al., 1996); others included positive urine toxicology for illicit drugs (n=2).

PPI was defined as (100-[100 × magnitude on prepulse trial/ magnitude on pulse alone trial]). Screening PPI across all 40 subjects was normally distributed (mean (SD) % across all intervals (10-120 ms) = 9.33 (20.25); median = 7.68; skewness = 0.27; kurtosis = 0.17). Startle magnitude, latency and PPI were analyzed with mixed-design ANOVAs, with trial type and pramipexole dose (placebo vs. active) as within-subject factors, and dose groups (i.e. subjects tested with placebo vs. 0.125 mg pramipexole ("0.125 mg group") and subjects tested with placebo vs. 0.1875 mg pramipexole ("0.1875 mg group")) as the between-subject factor. Separate analyses were then pursued on each dose group. No consistent drug interactions were noted with eye side (left vs. right), and thus main effects of eye side and interactions are not reported. "Baseline PPI" was defined as the mean %PPI for 60 and 120 ms prepulse intervals on the screening day, as described previously (Swerdlow et al., 2009b). Personality scales previously found to be related to drug effects on PPI were treated as continuous variables: the total novelty seeking score (NS) from the TPQ, total and disinhibition subscale scores from the SSS, and extraversion subscale scores from the EPQ. VAS ratings and autonomic measures were treated as continuous variables and were analyzed with mixed-design ANOVAs, focusing on difference scores (change from pre-pill baseline to time of PPI testing). Specific post-hoc comparisons were made with one-factor ANOVAs or the Fisher's Protected Least Significant Difference test (PLSD). Alpha was 0.05. In most cases, post-hoc comparisons were limited to tests of specific a priori hypotheses (e.g. that DA agonist effects on PPI would be dependent on baseline PPI) or planned comparisons (e.g. relationship of PPI pramipexole sensitivity to personality measures).

2. Rodent testing: The methods used in this study were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the UCSD Animal Subjects Committee (protocol #S01221). Adult male Sprague Dawley rats (225-250 g; Harlan Laboratories, San Diego, CA) were housed in groups of 2-3 animals per cage, and maintained on a reversed light/dark schedule with water and food available ad libitum. Rats were handled within 2 d of arrival. Testing occurred during the dark phase. Pramipexole (Toronto Research Chemicals, Inc., North York, Ontario, Canada) was dissolved in saline vehicle and administered subcutaneously in doses of 0 or 0.1 mg/kg (n=8), 0 or 0.3 mg/kg (n=8) or 0 or 1.0 mg/kg (n=8), with the same parallel within-subject dose design used in humans (doses from Weber et al., 2008a). Startle chambers (San Diego Instruments, San Diego, CA, USA) were housed in a sound-attenuated room, and consisted of a Plexiglas cylinder 8.2 cm in diameter resting on a 12.5 × 25.5 cm Plexiglas frame within a ventilated enclosure. Noise bursts were presented via a speaker mounted 24 cm above the cylinder. A piezoelectric accelerometer mounted below the Plexiglas frame detected and transduced motion from within the cylinder. Stimulus delivery was controlled by the SR-LAB microcomputer and interface assembly, which also digitized (0-4095), rectified, and recorded stabilimeter readings. One hundred 1-ms readings were collected beginning at stimulus onset. Startle amplitude was defined as the average of the 100 readings.

Approximately 7 days after shipment arrival, rats were exposed to a short "matching" startle session. They were placed in the startle chambers for a 5 min acclimation period with a 70 dB(A) background noise, and then exposed to a total of 17

P-ALONE trails (40 ms - 120 dB(A) noise bursts) that were interspersed with 3 PP12dB+P-ALONE trials (P-ALONE preceded 100 ms (onset-to-onset) by a 20 ms noise burst of 12dB above background). Rats were assigned to dose order groups based on average %PPI from the matching session to ensure similar baseline PPI levels between groups. Four days later, rats were injected with pramipexole (vehicle or active dose), and 15 min later placed in the startle chambers for a 5 min acclimation period with a 70 dB(A) background noise. They were then exposed to a series of trial types within a test session identical to that used in testing humans (see above, "Human testing"). Three days later, testing was repeated, with pramipexole dose reversed. Statistical analyses of startle magnitude and PPI were structured identically to those used in humans.

D. Results:

    1. Human testing: Demographic variables and personality scale scores for all subjects are seen in Table 1.1.

Table 1.1: Subject Characteristics

Subject characteristics	0.125 mg dose	0.1875 mg dose
Age (mean (range), years)	21.9 (18-30)	22.5 (18.0-33.0)
Weight (mean (range), kg)	76.8 (59.4-122.5)	72.3 (56.7-89.8)
Dose PRAM (mean (range), mg/kg $\times 10^3$)	1.67 (1.02-2.10)	2.63 (2.08-3.30)
Daily caffeine intake (mean (range), mg)	138.5 (0.0-615.2)	62.3 (0.0-280.0)
Personality scale scores (mean (range))		
TPQ		
Novelty seeking	17 (8-23)	15 (4-25)
Harm avoidance	8 (0-19)	8 (0-18)
Reward dependence	19 (9-27)	19 (10-26)
SSS		
Total score	21 (10-28)	20 (8-31)
EPQ		
Total score	21 (9-29)	20 (7-32)

Effects of pramipexole on autonomic and self-rating measures are seen in Figure 1.1 and Table 1.2. The only robust evidence of bioactivity of these doses of pramipexole came in significant increases in self-rated "drowsiness" (Figure 1.1). ANOVA of the change in ratings from pre-pill baselines for all subjects revealed a significant effect of pramipexole (F=8.16, df 1,38, p<0.007), no significant effect of dose group (F<1), and no significant interaction of pramipexole × dose group (F = 1.04). Analysis limited to 0.125 mg pramipexole revealed no significant effect of pramipexole on drowsiness (F = 2.94, df

1,19, ns), while analysis limited to 0.1875 mg pramipexole revealed a significant increase in drowsiness at the time of PPI testing (mean (SEM) increase in VAS after pramipexole = 23.6 (6.02) vs. placebo = 5.05 (5.50); F=5.27, df 1,19, p<0.035). In contrast, no significant effects of pramipexole were detected for either dose in measures of heart rate, blood pressure, pupil diameter, oral temperature or spontaneous blink rate, or in self-rated measures of dizziness, queasiness, happiness or sensory sensitivity (Table 1.2).

Pramipexole tended to diminish startle magnitude on pulse trials, but this effect did not reach statistical significance. Across all subjects, ANOVA of startle magnitude on all trial types revealed no significant main effect of pramipexole (F=2.94, df 1,38, 0.10 > p > 0.05) or dose group (F<1), and no pramipexole × dose group interaction (F = 2.12, df 1,38, ns). There was a significant effect of trial type (reflecting the startle-reducing effects of prepulses: F = 33.14, df 5,190, p<0.0001), but no other significant 2- or 3-way interactions. Inspection of the data (Figure 1.2) suggested no effect of 0.125 mg pramipexole on startle magnitude, and this was confirmed by ANOVA (placebo vs. 0.125 mg: F<1; main effect of trial type: F=15.42, df 5,95, p<0.0001; no significant 2-way interactions). However, 0.1875 mg pramipexole did appear to reduce startle magnitude, and this was also confirmed by ANOVA (placebo vs. 0.1875 mg: F = 5.40, df 1,19, p = 0.005; main effect of trial: F = 18.32, df 5,95, p<0.0001). The interaction of dose × trial type was also significant (F=2.43, df 5,95, p<0.045), suggesting different effects of pramipexole on startle magnitude across the different trial types. Post-hoc comparisons revealed that pramipexole did not significantly reduce startle magnitude on pulse alone trials (F=2.06, df 1,19, ns), but did significantly reduce startle magnitude on 120 ms

prepulse trials (F=11.97, df 1,19, p<0.003) (Figure 1.2A). To examine the independence of pramipexole effects on pulse alone vs. 120 ms prepulse trials, a subgroup of 17 subjects was created by eliminating 3 subjects whose pulse alone startle magnitude was most reduced by pramipexole. In this way, the remaining group (n=17) exhibited absolutely no startle-reducing effects of pramipexole on pulse alone trials (F<<1; mean (SEM) startle magnitude placebo vs. 0.1875 mg pramipexole = 117.20 (15.90) vs. 116.04 (17.75)), but nonetheless exhibited significant startle-reducing effects of pramipexole on 120 ms prepulse trials (F=8.46, df 1,16, p = 0.01) (Figure 1.3A).

Consistent with the selective startle-reducing effects of pramipexole on 120 ms prepulse trials, pramipexole significantly increased PPI at 120 ms intervals (Figure 1.2B). Across all subjects, ANOVA revealed no main effect of pramipexole (F<1) or dose group (F<1), and no pramipexole × dose group interaction (F < 1). There was a significant effect of trial type (F = 51.76, df 4,152, p<0.0001), and a significant interaction of pramipexole × dose group × trial type (F=2.53, df 4,152, p<0.05). Post-hoc comparisons revealed significant PPI-enhancing effects of pramipexole on 120 ms prepulse trials (main effect of pramipexole: F = 4.12, df 1,38, p<0.05). This effect did not differ by dose group (F<1), nor was there an interaction of pramipexole × dose group (F<1), though the PPI-enhancing effects of 0.1875 mg appeared to be more robust than those of 0.125 mg (d = 0.48 vs. 0.20). We examined whether the PPI-enhancing effects of 0.1875 mg pramipexole were independent of the modest startle-reducing effects of this dose on pulse alone trials, using the same subgroup of 17 subjects (described above) for whom this dose of pramipexole did not reduce pulse alone startle magnitude. The PPI-enhancing effects

of 0.1875 mg pramipexole were highly significant in this subgroup (F=10.53, df 1,16, p = 0.005; d = 0.79) (Figure 1.3B). Thus, pramipexole increased PPI at 120 mg prepulse intervals, particularly at the 0.1875 mg dose, and this effect was independent of changes in pulse alone startle magnitude.

Spearman Rank correlations assessed relationships between the PPI-enhancing effects of 0.1875 mg pramipexole at 120 ms prepulse intervals (calculated as a difference score: PPI (pramipexole) minus PPI (placebo)) and scores for novelty seeking (TPQ total NS scale), sensation seeking (SSS total score and DIS subscale score) and extraversion (EPQ-E subscale score). No correlations approached statistical significance ($-0.27 < R_s < 0.05$). Simple regression analyses revealed no correlation between these PPI-enhancing effects of pramipexole at 120 ms prepulse intervals and baseline startle magnitude (r = 0.02) or the impact of pramipexole on drowsiness (r = 0.03).

Pramipexole had no significant effects on startle latency, latency facilitation (reduction in latency caused by prepulses) or habituation (Table 1.3).

2. Rodent testing: The highest dose of pramipexole (1.0 mg/kg) reduced startle magnitude on pulse alone trials (Figure 1.4A). ANOVA of startle magnitude across all dose groups revealed no significant effect of group (F<1), a significant effect of pramipexole dose (vehicle vs. active dose) (F=10.91, df 1,21, p<0.004), and no interaction of group × dose (F=1.45 df, 2,21, ns). Post-hoc comparison revealed significant startle-reducing effects limited to the 1.0 mg/kg group (p<0.035).

Pramipexole had dose- and interval-dependent effects on PPI in rats (Figure 4B). ANOVA across all groups revealed near significant effects of group ($F = 3.32$, df 2,21, $p<0.056$) and dose ($F = 3.28$, df 1,21, $p<0.085$), a near-significant interaction of group $\times$ dose ($F = 3.24$, df 2,21, $p<0.06$), significant effects of prepulse interval ($F = 25.70$, df 4,84, $p<0.0001$), and a significant interaction of dose $\times$ interval ($F = 3.60$, df 4,84, $p<0.01$), but no significant 3-way interaction. In 3 rats from the high dose group, mean startle values on prepulse+pulse trials for some prepulse intervals greatly exceeded startle values on pulse alone trials, yielding large negative values for %PPI (i.e. prepulse potentiation), and thus accounting for the large error bars demonstrated in Figure 1.4B; capping these values (e.g. at -100%) did not alter the statistical outcome. Inspection of the data revealed dose-dependent PPI-reducing effects of pramipexole at the 120 ms prepulse interval, and PPI-increasing effects of lower doses of pramipexole at short (10-20 ms) prepulse intervals. Post-hoc comparisons for the 120 ms prepulse interval revealed significant effects of group ($F = 4.15$, df 2,21, $p<0.035$) and dose ($F = 15.04$, df 1,21, $p<0.001$), and a significant group $\times$ dose interaction ($F = 3.45$, df 2,21, $p = 0.05$). Pramipexole significantly reduced 120 ms PPI at the 0.3 mg/kg and 1.0 mg/kg doses ($p<0.008$ and 0.03, respectively), but not at the 0.1 mg/kg dose. Post-hoc comparisons for the 10-20 ms intervals revealed significant PPI-increasing effects of the 0.1 and 0.3 mg/kg doses (main effect of dose: $F = 6.81$, df 1,14, $p = 0.02$; no effect of group: $F<1$; no dose $\times$ group interaction: $F<1$).

While significant PPI-reducing and PPI-increasing effects of pramipexole were detected at doses that did not significantly reduce startle magnitude on pulse alone trials,

we nonetheless examined whether drug effects on startle magnitude and PPI could be more completely dissociated. This was done in two ways. First, difference scores (vehicle minus active dose) were calculated for pramipexole effects on pulse alone startle magnitude for each rat, and median splits were used to divide each dose group into rats with the least vs. most startle-reducing effects of pramipexole. Using this median split as a grouping factor, ANOVAs confirmed both the PPI-reducing effects of 0.3 and 1.0 mg/kg doses at 120 ms prepulse intervals, and PPI-enhancing effects of 0.1 and 0.3 mg/kg doses at 10-20 ms intervals, with no significant interactions of dose $\times$ median splits (all F's < 1.75, 1, 1 and 1, respectively). Next, simple regression analyses revealed no significant correlations between startle magnitude difference scores (i.e. the effect of pramipexole on startle magnitude) and PPI difference scores (i.e. the effect of pramipexole on PPI) at either the short (10-20 ms) or long (120 ms) prepulse intervals (r's < 0.30, ns).

Lastly, we examined the potential relationship between baseline PPI (during the dose-matching session) and pramipexole effects on PPI. Baseline PPI did not correlate significantly with the effect of pramipexole on PPI at either the short (10-20 ms) or long (120 ms) prepulse intervals (r's < 0.18, ns).

Table 1.2. Autonomic and subjective effects of pramipexole (mean (SEM))

	Min post-pill	Heart rate (BPM)	BP systolic (mm)	BP diastolic (mm)	Queasy (VAS)	Dizzy (VAS)	Can't focus (VAS)	Sounds loud (VAS)	Happy (VAS)	Pupil[a] (mm)	Temp[b] (°F)
					0.125 mg group						
	0[c]	**68.6 (1.4)**	**116.4 (1.7)**	**69.3 (1.5)**	**2.6 (1.0)**	**2.8 (1.2)**	**6.6 (3.5)**	**2.2 (0.9)**	**67.6 (6.0)**	**5.4 (0.2)**	**97.3 (0.1)**
Change after placebo	15	0.6 (0.8)	-1.3 (1.1)	-0.7 (0.7)	0.6 (1.1)	1.4 (1.0)	0.9 (0.8)	1.1 (0.7)	-1.1 (1.8)	-----	-----
	80	1.6 (1.1)	-1.6 (1.8)	1.8 (1.1)	1.8 (1.7)	3.4 (1.6)	1.2 (1.0)	1.0 (1.0)	-3.0 (2.1)	-----	-----
	110	2.6 (1.1)	-2.3 (1.6)	1.4 (1.3)	1.4 (1.5)	1.6 (1.7)	1.6 (0.9)	1.4 (1.4)	-4.8 (2.9)	-----	-----
	140	1.6 (1.1)	-3.0 (1.6)	1.0 (1.2)	1.2 (1.6)	2.3 (2.5)	0.1 (1.3)	0.8 (0.7)	-0.8 (2.2)	0.0 (0.2)	0.1 (0.1)
	220	0.4 (1.3)	-1.5 (1.7)	-1.7 (1.6)	-0.7 (1.1)	-0.6 (1.0)	1.8 (2.6)	1.1 (1.0)	-3.4 (2.7)	-----	-----
	255	1.4 (1.5)	-0.6 (1.6)	-0.7 (1.7)	-0.7 (1.2)	-0.2 (1.2)	0.6 (1.4)	0.8 (0.6)	-4.2 (3.4)	-----	-----
	445	-----	-----	-----	-----	-----	-----	-----	-----	0.3 (0.1)	0.4 (0.1)
	0	**67.4 (1.5)**	**114.1 (1.7)**	**69.0 (1.6)**	**2.0 (0.7)**	**2.6 (0.9**	**6.5 (3.0)**	**2.7 (1.0)**	**64.4 (6.5)**	**5.2 (0.2)**	**97.3 (0.1)**
Change after active dose	15	1.9 (0.9)	-1.6 (0.6)	-2.6 (0.9)	0.4 (0.5)	1.1 (1.1)	0.1 (1.6)	0.5 (0.6)	2.9 (2.4)	-----	-----
	80	1.8 (1.1)	-2.3 (0.9)	-0.6 (1.1)	1.5 (1.0)	1.7 (1.2)	1.1 (1.8)	5.0 (3.2)	-3.3 (3.4)	-----	-----
	110	0.8 (1.3)	-2.6 (0.9)	-0.3 (1.3)	1.9 (1.4)	4.8 (2.9)	0.7 (2.1)	1.8 (1.3)	-1.8 (4.4)	-----	-----
	140	1.0 (1.2)	-3.6 (1.1)	-1.0 (1.3)	1.3 (0.9)	2.1 (1.2)	1.1 (2.0)	1.2 (1.0)	-4.7 (3.2)	0.4 (0.2)	0.3 (0.1)
	220	3.8 (1.2)	0.3 (1.0)	-0.1 (1.2)	0.6 (0.6)	0.5 (0.6)	0.7 (2.0)	0.8 (1.0)	-0.7 (4.4)	-----	-----
	255	3.4 (1.2)	0.1 (1.0)	-1.1 (1.3)	1.2 (0.9)	0.4 (0.5)	-0.1 (1.7)	0.3 (0.6)	0.5 (3.6)	-----	-----
	445	-----	-----	-----	-----	-----	-----	-----	-----	0.7 (0.2)	0.4 (0.2)

Table 1.2. Autonomic and subjective effects of pramipexole (mean (SEM))(continued)

	Min post-pill	Heart rate (BPM)	BP systolic (mm)	BP diastolic (mm)	Queasy (VAS)	Dizzy (VAS)	Can't focus (VAS)	Sounds loud (VAS)	Happy (VAS)	Pupil[a] (mm)	Temp[b] (°F)
					0.1875 mg group						
	0	**65.6 (2.3)**	**112.9 (2.6)**	**64.7 (1.4)**	**1.9 (0.8)**	**1.5 (0.4)**	**1.2 (0.4)**	**5.8 (3.5)**	**87.9 (3.7)**	**5.5 (0.2)**	**97.5 (0.1)**
Change after placebo	15	2.8 (1.4)	-0.9 (1.0)	0.1 (1.2)	-0.2 (0.6)	4.9 (4.9)	0.2 (0.2)	-2.0 (1.3)	-1.4 (1.6)	-----	-----
	80	2.8 (1.5)	0.3 (1.2	1.3 (1.5)	-0.7 (0.8	4.2 (3.6	-0.1 (0.3)	-2.0 (1.1	-5.3 (2.5)	-----	-----
	110	3.2 (1.4)	0.2 (1.2)	1.8 (1.1)	-0.2 (0.2)	3.2 (2.9)	-0.2 (0.3)	-2.2 (1.3)	-4.8 (2.7)	-----	-----
	140	2.0 (1.8)	-0.1 (1.0)	2.5 (1.0)	-0.6 (0.7)	3.1 (3.3)	0.2 (0.3)	-1.7 (1.4)	-4.0 (2.7)	0.3 (0.2)	0.2 (0.2)
	220	2.6 (1.8)	0.6 (1.5)	2.3 (1.2)	-0.9 (0.6)	3.6 (4.0)	0.2 (0.3)	-2.1 (1.7)	-2.8 (3.1)	-----	-----
	255	2.6 (1.7)	0.2 (1.6)	1.5 (1.3)	-0.9 (0.6)	4.8 (4.9)	0.6 (0.3)	-2.3 (2.0)	-3.3 (3.6)	-----	-----
	445	-----	-----	-----	-----	-----	-----	-----	-----	-0.3 (0.2)	0.4 (0.1)
	0	**66.2 (1.7)**	**111.5 (2.4)**	**64.1 (1.6)**	**1.4 (0.3)**	**1.6 (0.6)**	**1.6 (0.4)**	**3.6 (2.3)**	**89.1 (4.1)**	**5.7 (0.3)**	**97.6 (0.2)**
Change after active dose	15	3.0 (1.0)	0.2 (1.6)	0.8 (1.8)	0.6 (0.3)	1.4 (0.9)	1.1 (0.7)	0.8 (0.7)	0.2 (1.2)	-----	-----
	80	3.0 (1.4)	-1.6 (1.3)	1.8 (1.6)	0.1 (0.4)	1.8 (1.3)	0.1 (0.5)	0.9 (0.5)	0.0 (1.3)	-----	-----
	110	3.2 (1.4)	-1.0 (1.6)	1.9 (1.9)	0.2 (0.3)	0.8 (0.5)	-0.4 (0.5)	1.1 (0.7)	-4.5 (2.1)	-----	-----
	140	1.6 (1.1)	-0.8 (1.6)	3.4 (2.1)	0.6 (0.4)	3.0 (2.5)	-0.2 (0.4)	0.6 (0.7)	-3.4 (2.1)	0.2 (0.3)	0.2 (0.1)
	220	4.2 (1.2)	1.7 (2.1)	1.0 (1.4)	0.2 (0.4)	3.1 (2.6)	-0.5 (0.4)	-0.2 (0.5)	-3.5 (2.4)	-----	-----
	255	3.9 (1.7)	4.2 (2.4)	4.5 (1.6)	0.5 (0.6)	4.4 (3.9)	-0.1 (0.3)	-2.0 (2.4)	-1.4 (2.3)	-----	-----
	445	-----	-----	-----	-----	-----	-----	-----	-----	-0.3 (0.3)	0.3 (0.2)

a. Diameter; b. oral temperature; c. Pre-pill baseline

Table 1.3. Peak reflex latency and habituation

	Peak Startle Latency (ms) (mean (SEM))			
	0.125 mg group		**0.1875 mg group**	
Prepulse Interval (ms)	**Placebo**	**Active dose**	**Placebo**	**Active Dose**
0*	60.9 (1.6)	61.4 (2.0)	63.5 (1.2)	62.1 (1.0)
10	56.9 (1.6)	58.3 (2.2)	59.7 (1.3)	59.3 (1.2)
20	55.9 (1.8)	56.7 (2.2)	59.7 (1.3)	58.5 (0.9)
30	54.2 (1.8)	55.9 (2.1)	56.6 (1.2)	57.4 (0.9)
60	52.5 (1.8)	54.0 (2.1)	54.0 (1.4)	54.9 (1.6)
120	57.2 (1.3)	56.9 (2.0)	58.6 (1.3)	59.1 (1.2)
	Startle Magnitude on Pulse Alone Trials (mean (SEM))			
First block	117.2 (16.0)	116.7 (17.9)	145.9 (19.2)	144.6 (19.2)
Last block	81.6 (14.7)	73.1 (16.9)	87.8 (16.6)	92.5 (19.5)

* pulse alone trials

Figure 1.1. Self-rated drowsiness VAS scores after placebo (open circles), 0.125 mg or 0.1875 mg pramipexole (closed circles), compared to pre-pill baselines (mean $\pm$ SEM). Time of PPI testing is indicated by arrow. # = significantly greater increase in drowsiness at time of PPI testing, pramipexole vs. placebo (p<0.007) by ANOVA; post-hoc individual comparisons detected significant effects only for the 0.1875 mg dose (p<0.035).

Figure 1.2. Effects of pramipexole on startle magnitude. (mean $\pm$ SEM) (A) and %PPI (mean $\pm$ SEM) (B) across all trial conditions in groups treated with placebo vs. 0.125 mg pramipexole (n=20) or placebo vs. 0.1875 mg pramipexole (n=20). Open circle = placebo; closed circle = active dose. * = startle reducing effects of 0.1875 mg pramipexole on prepulse+pulse trials at 120 ms prepulse intervals ($p<0.003$); # = PPI-enhancing effects of pramipexole at 120 ms prepulse intervals ($p<0.05$).

Figure 1.3. Effects of pramipexole on startle magnitude. (mean $\pm$ SEM) (A) and %PPI (mean $\pm$ SEM) (B) across all trial conditions in a subgroup of subjects treated with placebo vs. 0.1875 mg pramipexole (n=17) in whom pramipexole caused no change in pulse alone startle magnitude. Open circle = placebo; closed circle = active dose. * = startle reducing effects of pramipexole on prepulse+pulse trials at 120 ms prepulse intervals (p=0.01); # = PPI-enhancing effects of pramipexole at 120 ms prepulse intervals (p=0.005).

Figure 1.4. Effects of pramipexole on startle magnitude (inset; mean $\pm$ SEM) and %PPI (mean $\pm$ SEM) across all trial conditions in 3 groups of rats administered placebo vs. 0.1 mg/kg pramipexole (n=8), placebo vs. 0.3 mg/kg pramipexole (n=8) or placebo vs. 1.0 mg/kg mg pramipexole (n=8). Open circle = placebo; closed circle = active dose. Insert: * = startle reducing effects of 1.0 mg/kg pramipexole (p<0.035). Main Figure: # = PPI-enhancing effects of pramipexole at 10-20 ms prepulse intervals for 0.1 and 0.3 mg/kg doses (p=0.02); * = PPI-reducing effects of 0.3 and 1.0 mg/kg pramipexole (p<-0.008 - 0.03).

E. Discussion:

In the present study, pramipexole, a D3-preferential agonist, increased long interval PPI in clinically normal men, and had biphasic dose effects on PPI in rats - increasing short interval PPI at low doses, and decreasing long interval PPI at higher doses. Unlike the PPI-enhancing or reducing effects of the indirect DA agonist amphetamine (Hutchison et al., 1999; Talledo et al., 2009), the effects of pramipexole were not moderated by personality measures of novelty seeking, sensation seeking, disinhibition or extraversion; unlike the PPI-modulatory effects of either amphetamine (Swerdlow et al., 2003b; Talledo et al., 2009), direct D2/D3 agonists (Bitsios et al., 2005), or the NMDA antagonist/ DA releaser memantine (Swerdlow et al., 2009b), these effects of pramipexole were independent of baseline PPI levels.

The fact that pramipexole effects on PPI differ from those of other DA agonists is consistent with preclinical evidence in rats and humans. In rats, PPI-reducing effects of pramipexole do not differ across strains that exhibit pronounced differences in sensitivity to the PPI-disruptive effects of amphetamine (Swerdlow et al., 2003b; Talledo et al., 2009) and mixed D2/D3 agonists (Swerdlow et al., 2001c, 2004a, 2004b, 2004c; Weber et al., 2008b). Also, unlike amphetamine and mixed D2/D3 agonists, the PPI-disruptive effects of pramipexole in rats are relatively insensitive to selective D2 receptor blockade, but are opposed by compounds with selective functional antagonism at D3 receptors (Weber et al., 2009b). Evidence for differences in the PPI-modifying effects of pramipexole vs. other DA agonists in humans comes from findings that personality dimensions and/or baseline PPI moderate the PPI-disruptive effects of

amphetamine (Hutchison et al., 1999; Talledo et al., 2009) and mixed D2/D3 agonists (Bitsios et al., 2005), but not those of pramipexole (present study); this may suggest that these moderating effects primarily reflect activity at D2- and not D3 DA receptors.

Pramipexole-induced increases in humans were limited to the 120 ms prepulse intervals. Reflex inhibition at these longer intervals can be enhanced by attention directed at a continuous prepulse (Filion et al., 1993). While the present study utilized discrete (vs. continuous) prepulses, and no attentional instructions were given, it is nonetheless possible that the mechanisms responsible for pramipexole's enhancement of "attentionally-sensitive" inhibition reflect its action on brain mechanisms that regulate attention. No significant correlations were detected between increases in self-rated drowsiness and PPI, but the present VAS measures may not be adequately sensitive to detect meaningful inter-individual differences in alertness, and are not designed to assess attentional states. In a recent study with 10 clinically normal adults, higher doses of pramipexole (0.25 - 0.50 mg) caused sedation and impaired cognitive performance in sedation-sensitive tasks (Hamidovic et al., 2008). However, because directed attention is associated with *higher* PPI levels in normal humans (Filion et al., 1993), it would be counter-intuitive to explain the observed PPI *increases* based on attention-*impairing* effects of pramipexole.

Confirmation that the present doses of pramipexole were bioactive came from increases in self-rated drowsiness, consistent with a number of reports with higher doses of pramipexole (Hamidovic et al., 2008; Samuels et al., 2006a, 2006b, 2007). The lack of pramipexole effects on measures such as "queasiness", "happiness", and on

autonomic measures (e.g. pupil dilation), suggests that the doses in this study were physiologically low (Hamidovic et al., 2008; Samuels et al., 2006a, 2006b, 2007), and raises the possibility that different effects on PPI might have been detected at higher doses. Such a prediction would be consistent with the present study in rodents, which demonstrated an inverted-U dose function for pramipexole's effects on PPI (i.e. it increased PPI at low doses and reduced PPI at high doses). Conceivably, low doses of pramipexole might preferentially activate presynaptic receptors, or a combination of pre- and post-synaptic receptors, making it difficult to determine whether the observed increase in PPI reflected functional decreases or increases in forebrain D3 stimulation (Samuels et al., 2006b).

The nature of most studies using systemic drug administration is that the precise mechanisms of drug effects remain a matter of speculation. If we posit that the PPI-enhancing effects of pramipexole in humans reflect activation of post-synaptic forebrain D3 receptors, it would be difficult to account for PPI *deficits* observed in schizophrenia (Braff et al., 1978) or several other neuropsychiatric disorders (cf. Braff et al., 2001, Swerdlow et al., 2008b) based solely on overactivity of D3 neurotransmission. On the other hand, if we posit that these effects of pramipexole reflect its action at presynaptic DA receptors that reduce forebrain DA "tone" (Samuels et al., 2006b), then the observed increases in PPI might be loosely consistent with PPI-enhancing effects of DA receptor antagonists in previous reports (Csomor et al., 2008; Swerdlow et al., 2006c; Vollenweider et al., 2006). A strong caveat to such a connection - between PPI-enhancement by putative presynaptic effects of pramipexole,

and post-synaptic receptor-blocking effects of antipsychotics - is that these effects of antipsychotics are moderated by both personality dimensions (Swerdlow et al., 2006c) and baseline PPI (Csomor et al., 2008; Vollenweider et al., 2006), neither of which moderate the present effects of pramipexole. Certainly, one might imagine that the PPI-enhancing effects of drugs acting pre- vs. post-synaptically could both reflect reduced DAergic "tone", yet be moderated by different biological factors. It is also conceivable that different patterns of pramipexole effects might have emerged had the 40 subjects in this study been stratified based on different polymorphisms for the D3 receptor (Roussos et al., 2008a).

Absent a clear understanding of whether the present drug effects reflect pre- vs. post-synaptic actions of pramipexole (or some combination thereof), and lacking genetic information on D3 receptor polymorphisms among our subjects, we can still conclude from the present data that: 1) D3 receptors do appear to regulate PPI in clinically normal humans; 2) activation of these receptors by low doses of pramipexole enhances long-interval PPI; and 3) these effects appear to be independent of physiological variables that moderate other forms of DAergic regulation of PPI. It would be reasonable to consider whether such an effect could be used as a basis for predicting therapeutic sensitivity to D3 stimulation in clinical populations.

F. Acknowledgements:

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CHAPTER 2:

**Parametric approaches towards understanding the effects of the preferential D3
receptor agonist pramipexole on prepulse inhibition in rats**

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A, Abstract:

The preferential dopamine D3 receptor agonist pramipexole (PRA) disrupts
prepulse inhibition (PPI) of acoustic startle, an operational measure of sensorimotor
gating, in rats. Drug effects on PPI are sensitive to numerous experimental variables;
proceeding with in-depth analyses of drug effects without a clear understanding of these
variables is inefficient. The present studies characterized the impact on PRA-induced PPI
deficits by a range of experimental parameters. As shown previously, PRA reduced both
PPI and startle magnitude beginning 5-15 min post-injection; PRA effects on PPI were
statistically significant through 35 min post-injection, while those on startle magnitude
were still significant 65 min post-injection. PRA-induced PPI deficits were evident under
conditions that matched startle magnitude in vehicle and PRA conditions and were
independent of PRA-induced changes in prepulse-elicited motor activity. Additionally,
PRA-induced PPI deficits did not differ significantly between uni- vs. cross-modal
stimuli or between male vs. female rats, with no robust effect of estrous phase in females.
These findings demonstrate that PRA effects on PPI are observed across several different

experimental conditions and are dissociable from changes in startle magnitude or prepulse-elicited responses. Recommendations are made regarding "optimal" experimental conditions for studying the neurobiology of PRA-induced changes in PPI in rats.

B. Introduction:

PPI is an operational measure of sensorimotor gating that is disrupted in several neuropsychiatric disorders, including schizophrenia and Tourette Syndrome (Castellanos et al., 1996; Swerdlow et al., 2001b, 2008b). PPI has been widely used in animal models to predict clinical efficacy of novel antipsychotic compounds. Recently, we reported that pramipexole (PRA) disrupts prepulse inhibition (PPI) of acoustic startle in rats (Weber et al., 2008a, 2009b). PRA is a non-ergot full agonist of dopamine D2 and D3 receptor subtypes, with selectivity for D3 over D2 receptors reported to be between 7:1 and 160:1 (Millan et al., 2002; Piercey et al., 1996; Svensson et al., 1994a). The dopamine D3 receptor (D3R) is of particular interest in the pathophysiology and treatment of several neuropsychiatric disorders, including schizophrenia, Tourette Syndrome, substance dependence, and depression. The selectivity of PRA for D3 over D2 receptors makes it an important pharmacological tool for studying the effects of D3R activation.

PPI and PPI response to drug treatments are sensitive to many different experimental parameters. Historically, studies of the neurobiology of these drug effects often have preceded studies that clarified the optimal experimental parameters; this

sometimes led to interpretative difficulties (Conti et al., 2009; Davis, 1988; Davis et al., 1990; Kinney et al., 1999; Palmer et al., 2000; Swerdlow et al., 1998a, 2008b). A more efficient strategy is to clarify the parametric sensitivity of drug effects in advance of embarking on complex neurobiological studies. We previously reported PRA dose-response effects on PPI and reported that Sprague-Dawley and Long-Evans rat strains display differential sensitivity to the nonselective dopamine agonist apomorphine, but not PRA (Weber et al., 2008a). We have also shown that PRA effects on PPI are sensitive to prepulse intervals (time between prepulse and pulse onset; Swerdlow et al., 2009a). The studies reported here employ parametric approaches to explore the relationship between PPI-disruptive effects of PRA and time course, startle reduction, prepulse elicited reactions, stimulus modalities, estrous phase, and sex.

C. Methods:

1. Experimental Animals: Adult Sprague-Dawley male (n = 56, 225-250 g; Harlan Laboratories, Livermore, CA) and female (n = 16, 175-200 g) rats were housed in same sex groups of 2-3 animals per cage, and maintained on a reversed light/dark schedule with water and food available *ad libitum.* Rats were handled within 2 d of arrival. Testing occurred during the dark phase. Males and females were housed in separate rooms and tested on different days. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, revised 1985) and were approved by the UCSD Animal Subjects Committee (protocol #S01221).

2. Drugs: Pramipexole (PRA) was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Drug doses are based on milligram/kilogram salts. PRA (0, 0.3, 1.0 mg/kg) was injected subcutaneously in a volume of 1 ml/kg body weight 15 min prior to behavioral testing, except in the time course experiment, in which there was no pretreatment time. Pseudorandom balanced dose orders were used.

3. Apparatus: Startle chambers for rats (San Diego Instruments, San Diego, CA, USA) were housed in a sound-attenuated room, and consisted of a Plexiglas cylinder 8.2 cm in diameter resting on a 12.5×25.5 cm Plexiglas frame within a ventilated enclosure. Noise bursts were presented via a speaker mounted 24 cm above the cylinder. Visual stimuli consisted of flashes of incandescent white light delivered via a 15 W light bulb. The light bulb was mounted to the ceiling of the chamber in a corner of the startle chamber at a distance of approximately 22 cm from the center of the rat cylinder. Light flashes did not generate any audible sound. A piezoelectric accelerometer mounted below the Plexiglas frame detected and transduced motion from within the cylinder. Stimulus delivery was controlled by the SR-LAB microcomputer and interface assembly, which also digitized (0-4095), rectified, and recorded stabilimeter readings. One hundred 1-ms readings were collected beginning at stimulus onset. Startle amplitude was defined as the average of the 100 readings.

4. Startle testing procedure: Approximately 7-9 d after shipment arrival, rats were exposed to a short "matching" startle session. They were placed in the startle chambers for a 5 min acclimation period with a 70 dB(A) background noise, and then exposed to a total of 17 P-ALONE trials (40 ms – 120 dB(A) noise bursts) that were interspersed with 3 PREPULSE+PULSE trials in which P-ALONE was preceded 100 ms (onset-to-onset) by a 20 ms noise burst, 12 dB above background. Rats were assigned to drug dose groups based on average %PPI from the matching session to ensure similar baseline PPI levels between groups. Starting 2-5 d after the matching session, drug testing began. Inter-test interval was 4-7 d. Drug testing was done on multiple days with PRA as a within-subjects factor. Test sessions varied by experiment (described below), but included P-ALONE and PREPULSE+PULSE trial types presented in pseudorandom order. Interspersed between consecutive active trials were NOSTIM trials in which activity was recorded, but no stimulus was presented. Prepulses preceded pulses by 100 ms (onset-to-onset), and average ITI between active trials was 15 s.

Specific parameters in different test sessions are described below:

a. "Time Course Session": The test session was divided into six 10-min blocks. Prepulses were 80 dB(A) (10 dB above background) and pulses were 120 dB(A). PREPULSE-ALONE active trials were also interspersed throughout the session and consisted of noise bursts 10 dB above background. Session duration was 65 min, including the initial 5 min acclimation period.

b. "Pulse Intensity Session": Prepulses were 3, 5, or 10 dB above background and preceded either 105 dB(A) or 120 dB(A) pulses. Session duration was approximately 23 min.

c. "Cross-Modal Session": 10 dB acoustic or 40 ms light flashes preceded 120 dB(A). PREPULSE-ALONE active trials were also interspersed throughout the session and consisted of noise bursts 10 dB above background. Session duration was approximately 15 min.

d. "Sex Differences Session": 5, 10, or 15 dB prepulses preceded the 120 dB(A) pulse by 100 ms. Session duration was approximately 18.5 min.

5. Estrous phase determination: (Adapted from Marcondes et al., 2002) Vaginal secretion was collected with a cotton swab wetted with normal saline (NaCl 0.9%) and placed on glass slides. Unstained material was viewed under a light microscope without the use of a condenser lens, with 10× and 40× objective lenses. Based on cell type distribution, animals were classified as being in proestrus, estrus, metestrus, or diestrus. Vaginal lavage and estrous phase determination were performed for 8 d prior to matching session to reduce the stress of the procedure on the animals and ensure normal cycling and average cycle length of 4 d. Vaginal lavage was performed immediately after each startle testing session, and test sessions were spaced 4 d apart.

6. Data Analysis: PPI was defined as 100-[(startle amplitude on PREPULSE trials/startle amplitude on PULSE-ALONE trials) × 100], and was analyzed by mixed design ANOVAs. All data were inspected for the presence of "non-responders" defined by a mean startle response to PULSE-ALONE trials of < 10 units; none met this criteria. Other ANOVAs were used to assess responses on PULSE-ALONE, PREPULSE-ALONE, or NOSTIM trials. Where different prepulse intensities were present, data were collapsed across all prepulse intensities, unless otherwise noted. Post-hoc comparisons were conducted using Fisher's protected least significance difference (PLSD). Difference scores for PPI and startle magnitude were calculated by subtracting values from each rat on drug-treatment days from vehicle-treatment days. Correlations between difference scores for PPI scores and startle magnitude were conducted using Spearman Rank Correlation. Alpha was set at 0.05.

D. Results:

1. Time course of effects: Rats treated with vehicle or PRA 1.0 mg/kg were immediately placed in startle chambers after injections for a 5-min acclimation, and responses were then measured over the course of a 60 min test session, divided into six 10-minute blocks. Repeated measures ANOVA of %PPI showed a significant main effect of PRA 1.0 mg/kg (F=80.61, *df* 1,7; *p*<0.0001), but no effect of time block (F=1.00, *df* 5,35; NS), and no PRA × block interaction (F=1.27, *df* 5, 35; NS) (Fig. 2.1A). Fisher's PLSD revealed that %PPI was significantly lower in PRA-treated rats during the first, second, and third 10-min blocks (*p*<0.025, 0.001, 0.0005, respectively). %PPI of PRA-

treated rats was significantly lower during the third block compared to the first block ($p<0.05$) and the fourth block ($p<0.05$).

ANOVA of startle response on P-ALONE trials revealed significant main effects of PRA (F=21.69, *df* 1,7; $p<0.003$), block (F=6.14, *df* 5,35; $p<0.005$), and a significant PRA × block interaction (F=5.24, *df* 5,35; $p<0.002$) (Fig. 2.1B). Fisher's PLSD showed that startle magnitude was significantly lower in PRA treated rats during blocks 1, 2, 3, and 6 ($p<0.002$, 0.0001, 0.0005, 0.02, respectively). Although the difference in startle magnitude between PRA- and vehicle-treated rats failed to reach significance during blocks 4 and 5, this was likely due to a decrease in startle magnitude in the vehicle group during these blocks. Indeed, post-hoc tests did not reveal a significant difference between any blocks among PRA-treated rats, but among vehicle-treated rats, startle magnitude for blocks 4 and 5 were significantly lower than that of block 1 ($p<0.04$) and block 2 ($p<0.006$).

Figure 2.1: Time course of PRA effects on %PPI (A) and startle magnitude (B).
A: PRA 1.0 mg/kg significantly reduced PPI during the first 30 minutes of testing,
with maximal effects during the third 10-minute block. (*, p<0.03) B: Startle-
reducing effects of PRA persist across 60 min. This difference was significant during
blocks 1,2,3, and 6. A decrease in startle magnitude during blocks 4 and 5 of the
vehicle condition likely account for the lack of significance of PRA effects during
these time periods.

2. PRA effects on PPI using weak and intense pulses: To match startle
magnitude between vehicle and PRA treatment groups, animals were tested in a session
that included trials with 120 dB(A) and 105 dB(A) pulse intensities, presented alone or
preceded by a prepulse. ANOVA of %PPI revealed significant main effects of PRA dose
(F=14.11, *df* 1,15; *p*<0.002) and pulse intensity (F=5.24, *df* 1,15; *p*<0.04), and a

significant PRA $\times$ pulse intensity interaction (F=6.26, *df* 1,15; *p*<0.03) (Fig. 2.2A). This interaction reflected significant PPI-disruptive effects of PRA with 120 dB(A) pulses, but not with 105 dB(A) pulses. As expected, there was also a significant main effect of prepulse intensity (F=11.43, *df* 2,30; *p*<0.0005), but no significant PRA $\times$ prepulse intensity or pulse intensity $\times$ prepulse intensity interactions (F=2.91, *df* 2,30; NS and F=3.19, *df* 2,30; NS, respectively). Data were collapsed across prepulse intensity for post-hoc analyses of PPI. ANOVA of startle response to P-ALONE revealed significant main effects of PRA (F=27.84, *df* 1,15; *p*<0.0001), pulse intensity (F=37.45, *df* 1,15; *p*<0.0001), and a significant PRA $\times$ pulse intensity interaction (F=6.33, *df* 1,15; *p*<0.03) (Fig. 2.2B). Importantly, Fisher's PLSD revealed that startle magnitude was not significantly different between vehicle-treated rats after "low intensity" pulses and PRA-treated rats after "high-intensity" pulses (Fig. 2.2A). Post-hoc comparisons of %PPI in these two conditions with matched startle magnitude showed that PPI was significantly reduced in PRA- vs. vehicle-treated rats (*p*<0.0001) (Fig. 2.2B).

Figure 2.2: Effects of PRA on %PPI in trials matched for startle amplitude. A: Startle magnitude was matched between vehicle-treated rats after "low intensity" 105 dB(A) pulses and PRA-treated rats after "high intensity" 120 dB(A) pulses. B: Under conditions in which startle magnitude was matched, PRA 1.0 mg/kg significantly reduced PPI (***, $p<0.0001$)
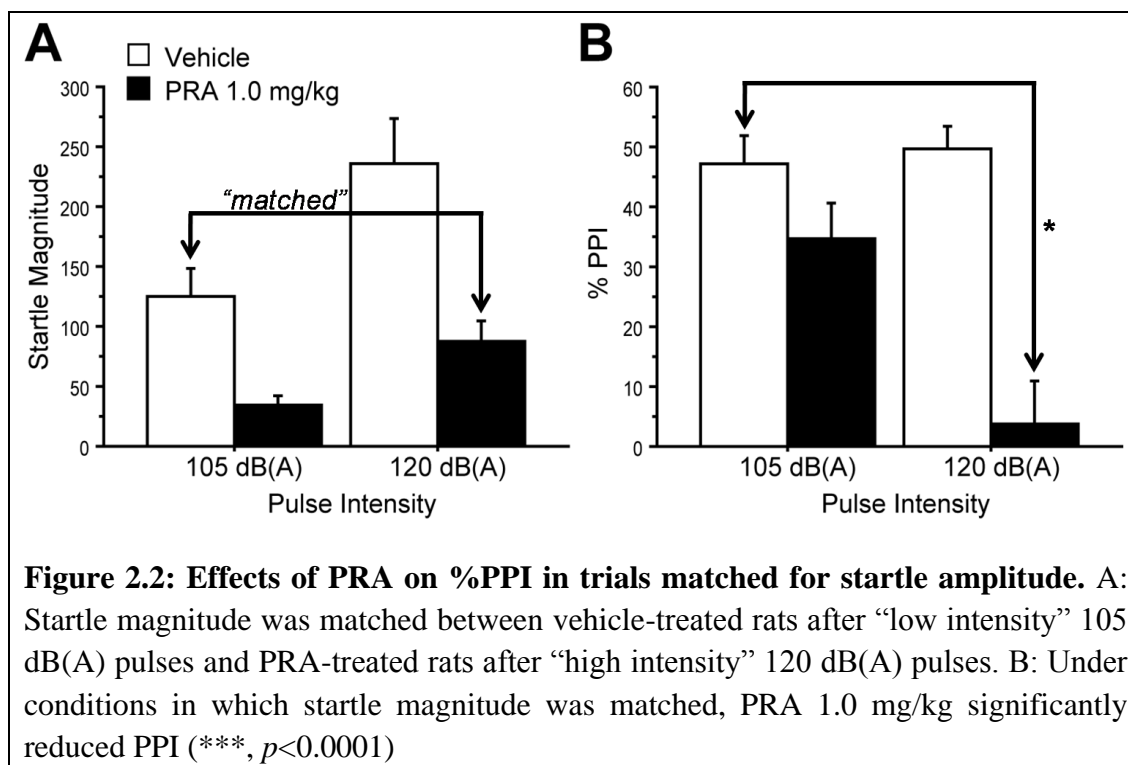
3. PRA effects on motor responses to prepulses: PREPULSE-ALONE trials were interspersed throughout the time course and cross-modal test sessions described above in order to measure prepulse-elicited responses (PPER). ANOVA of response to PREPULSE-ALONE during the time course session did not reveal any significant main effects of PRA (F=1.720, *df* 1,7; NS) or time block (F<1, *df* 5,35; NS), and no PRA × block interaction (F<1, *df* 5,35; NS) (mean (SEM) PPER, vehicle vs. PRA: 3.88 (2.02) vs. 0.58 (0.25)). There was a high degree of variability in the vehicle group during blocks 1-3 (Average response (SEM) for Block 1=8.583 (8.019), Block 2=6.000 (5.117), Block 3=7.875 (7.875)), due to a single outlier value in each of these blocks from one of two different animals. Responses for these same two animals during NOSTIM, PULSE-

ALONE, and PREPULSE+PULSE trials were not outside the range of the rest of the group. ANOVA of response to acoustic PREPULSE-ALONE during the cross-modal test also failed to reveal a significant main effect of PRA (F<1, *df* 2,30; NS) (mean (SEM) PPER, vehicle vs. PRA 0.3 mg/kg vs. PRA 1.0 mg/kg: 1.70 (0.78) vs. 1.19 (0.56) vs. 1.89 (0.45)).

4. Cross-modal PPI: ANOVA of %PPI revealed significant main effects of PRA dose (0, 0.3, 1.0 mg/kg) (F=8.59, *df* 2,30; $p<0.002$) and prepulse modality (F=13.46, *df* 1,15; $p<0.003$), but no significant PRA $\times$ prepulse modality interaction (F=2.84, *df* 2,30; NS) (Fig. 2.3). Fisher's PLSD showed a significant effect of both doses of PRA on acoustic PPI (PRA 0.3 mg/kg $p<0.004$, PRA 1.0 mg/kg $p<0.0001$), and a significant effect of 1.0 mg/kg PRA on visual PPI ($p<0.03$). Effects of the lower dose of PRA failed to reach significance for visual PPI, and inspection of the data (Fig. 2.3) suggested more robust effects of PRA on PPI when unimodal acoustic stimuli were used.

ANOVA of startle magnitude to PULSE-ALONE revealed a significant main effect of PRA (F=11.18, *df* 2,30; $p<0.0005$) (data not shown). Difference scores between vehicle treatment day and drug treatment day (either 0.3 or 1.0 mg/kg PRA) were calculated for startle magnitude and acoustic and visual PPI scores. Difference scores for PPI and startle magnitude were compared through Spearman Rank Correlation, and no significant correlations between PRA effects on startle magnitude and PPI were found

(range of $\rho=0.03$-0.30, all NS). Thus, drug effects on PPI were separable from drug effects on startle magnitude.

Figure 2.3: Effects of PRA on PPI with uni- and cross-modal stimuli. PPI with acoustic or visual (light) prepulses showed sensitivity to PRA (0, 0.3, 1.0 mg/kg). (*, *p*<0.03; **, *p*<0.004; ***, *p*<0.0001)
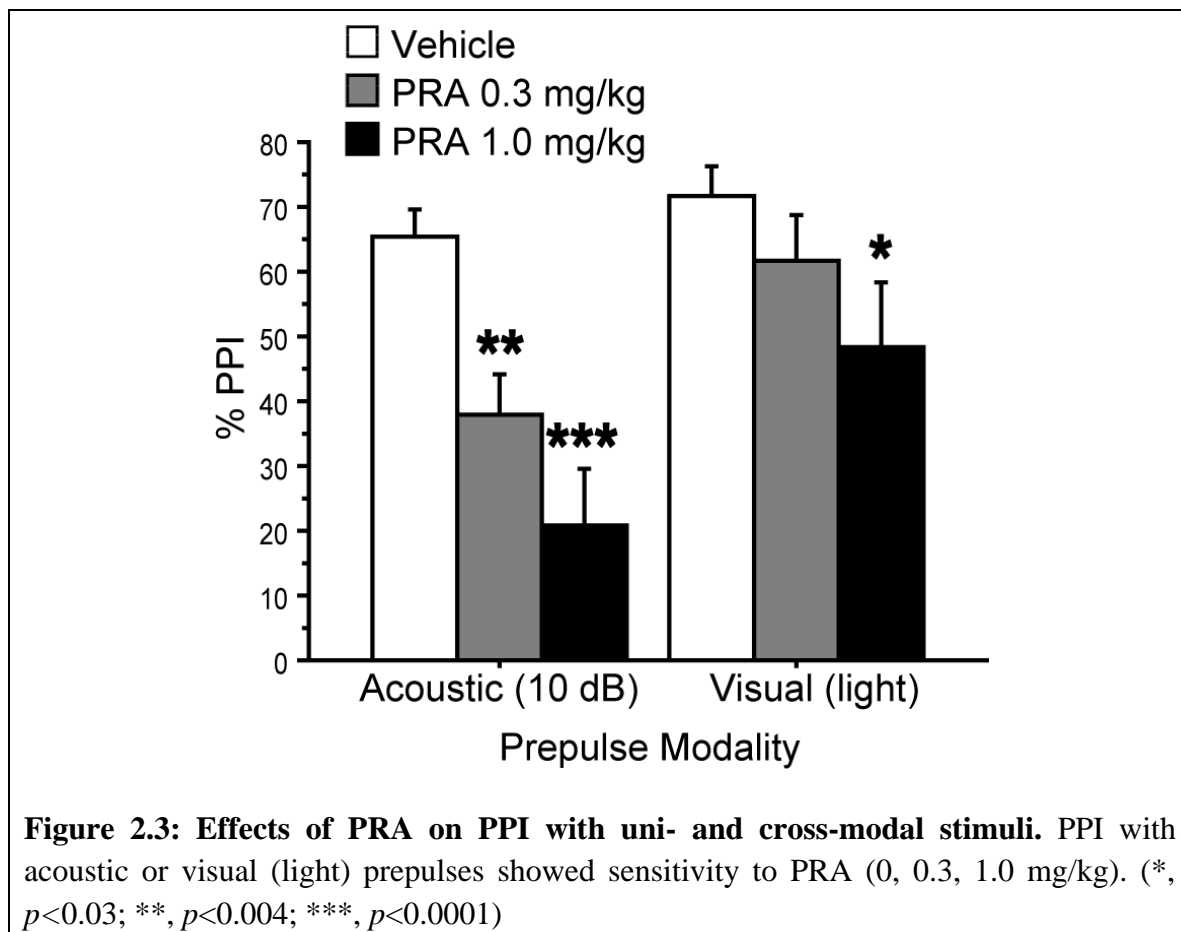
5. Effects of PRA on PPI across the estrous cycle: Some females displayed prolonged cycle length, but all showed progression through different phases of the cycle during this observation period. Test sessions were spaced 4 days apart to minimize the amount of phase variability for each rat between test days. However, estrous phase was determined after each PPI testing session, and not all cells of a dose × estrous phase comparison were filled. Due to phase cycles that were slightly more or less than 4 days

long, many rats were in the same phase for only two out of three drug testing days. This precluded a definitive within-subject assessment of PRA sensitivity across the estrous cycle. Qualitatively, within a given estrous phase, rats tested at two different doses of PRA had reduced %PPI with the higher dose relative to the lower dose. As there was a significant effect of prepulse intensity (F=12.44, *df* 2,30; *p*<0.0002) but no prepulse intensity × PRA dose interaction (F=2.10, *df* 4,60; NS), data were collapsed across prepulse intensity for further statistical analyses. Inspection of the data for rats that were in the same estrous phase for two different test days revealed a significant effect of PRA higher dose vs. lower dose (F=14.10, *df* 1,9; *p*<0.005), but no effect of estrous phase (F<1, *df* 2,9; NS) and no dose × phase interaction (F<1, *df* 2,9; NS). This lack of estrous phase effect or dose × phase interaction was true whether the lower dose was the vehicle or PRA 0.3 mg/kg condition. A similar analysis of startle magnitude between conditions of higher vs. lower PRA doses showed a significant effect of PRA dose (F=10.85, *df* 1,9; *p*<0.01), but no significant effect of estrous phase (F<1, *df* 2,9; NS) and no dose × phase interaction (F<1, *df* 2,9; NS). Again, there was no significant effect of estrous phase or dose × phase interaction whether the lower dose was vehicle or PRA 0.3 mg/kg.

6. Sex Differences: Data from age-matched male rats were combined with data from the estrous cycle study to assess sex differences in PRA effects on PPI (Fig. 2.4). ANOVA of %PPI showed a significant main effect of PRA dose (0, 0.3, 1.0 mg/kg) (F=33.07, *df* 2,60; *p*<0.0001), but no significant effect of sex (F=2.77, *df* 1,30; NS) and no PRA × sex interaction (F=1.33, *df* 2,60; NS). There was a significant effect of

prepulse intensity (F=33.39, *df* 2,60; *p*< 0.0001) but no prepulse intensity × PRA dose interaction (F=1.59, *df* 4,120; NS). Data were collapsed across prepulse intensity for post-hoc analyses. As had been found in females, post-hoc tests in males indicated that both 0.3 and 1.0 mg/kg PRA had a significant effect on PPI (*p*<0.002, 0.0001, respectively). However, post-hoc tests in females revealed a significant difference between the low and high doses of PRA (*p*<0.002), while these two conditions were not significantly different in males. Therefore, females exhibited a step-wise decrease in PPI with increasing doses of PRA, while males achieved a maximal effect with the lower dose of PRA.

ANOVA of startle magnitude across male and female groups revealed a significant main effect of PRA dose (F=18.22, *df* 2,30; *p*<0.0001). In contrast to PPI data, there was a significant main effect of sex (F=7.19, *df* 1,30; *p*<0.02), but no PRA × sex interaction (F<1, *df* 2,60; NS). ANOVAs comparing startle magnitude at each drug condition indicated that the sex difference was due to significantly lower startle magnitude in females compared to males in both active drug conditions, but not in the vehicle condition (Vehicle: F=1.35, *df* 1,30; NS; PRA 0.3 mg/kg: F=19.40, *df* 1,30; *p*=0.0001; PRA 1.0 mg/kg: F=13.30, *df* 1,30; *p*=0.001).

Again, PPI and startle magnitude difference scores were calculated for each rat based on vehicle and PRA treatment days. Spearman Rank Correlations, split by sex, did not indicate any correlation between PRA effects on PPI and startle magnitude at either dose (range of ρ= -0.17-0.28, all NS), suggesting that drug effects on PPI were separable from drug effects on startle magnitude.

Figure 2.4: PRA effects on %PPI (A) and startle magnitude (B) in male and female rats. A: Both sexes exhibited dose-dependent reductions in PPI after treated with PRA (0, 0.3, 1.0 mg/kg), though the difference between PPI in low- and high-dose conditions was significant in females but not males. (**, $p<0.003$; ***, $p<0.0001$) B: PRA (0, 0.3, 1.0 mg/kg) reduced startle magnitude in both males and females. There was a significant effect of sex ($p<0.02$), with lower startle magnitude in females than males with both active drug doses.

E. Discussion:

Compared to the D2 receptor, relatively little is known about the behavioral and cellular/molecular effects of D3 receptor activation or inhibition. The recent emergence of drugs like PRA, which are preferential for D3 versus D2 receptors, has facilitated the

study of D3 receptor systems in behavioral models. DA agonist effects on PPI are sensitive to many experimental variables, such as stimulus parameters (Mansbach et al., 1988; Swerdlow et al., 2009a; Weber and Swerdlow, 2008), rat strain (Conti et al., 2009; Swerdlow et al., 2004a, 2004b; Weber et al., 2008b; Weber and Swerdlow, 2008), sex (Lehmann et al., 1999; Swerdlow et al., 2008a) and estrous phase (Kinkead 2008; Koch, 1998), but most studies of the neurobiology of DA agonist effects on PPI proceeded without the benefit of knowing about these modifying experimental variables. The current studies were designed to "fill in the gaps" regarding parametric effects on PRA-induced PPI deficits in rats in advance of more detailed studies of the neurobiology of D3R effects on PPI.

PRA demonstrated significant effects on PPI during the first 30 min after systemic administration, with maximal effects during the third 10-minute block. The PRA-induced reduction in startle magnitude remained relatively constant across the one-hour test. In order to separate the startle-reducing effects from PRA-induced PPI deficits, startle magnitude was matched between vehicle- and PRA-treated rats by using startling pulses with 120 dB(A) and105 dB(A) intensities. Startle magnitudes in response to 105 dB pulses in vehicle-treated rats were very comparable to startle magnitudes in response to 120 dB pulses in PRA-treated rats; under these conditions of matched startle magnitude, PRA still significantly reduced PPI, suggesting that PRA-induced PPI deficits are independent of PRA effects on startle magnitude. This conclusion is further supported by a lack of correlation between startle magnitude and PPI difference scores, at two doses of PRA with uni- and cross-modal stimuli, as well as with acoustic stimuli in both males and

females. The present studies also revealed that the PPI-disruptive effects of PRA were most evident when startle pulses were intense (120 dB) vs. weak (105 dB), despite the fact that post-vehicle levels of PPI were comparable with 120 vs. 105 dB pulses. PRA effects on PPI were also dissociated from those on prepulse-elicited reactions, which have been suggested by some to be relevant to drug-induced changes in PPI (Yee and Feldon, 2009).

PRA-induced PPI deficits were evident using both acoustic and visual (light) prepulses with acoustic startle pulses. This is important because it confirms that the PPI-disruptive effects of PRA are mediated at a point in the nervous system that receives integrated visual and auditory information, rather than within primary sensory circuitry. Dose-response effects were observed in both uni- and cross-modal conditions. Apomorphine, a non-selective D1 and D2-like receptor agonist, has also been found to decrease PPI with both acoustic and visual prepulses (Campeau and Davis 1995, Weber and Swerdlow 2008), and PPI deficits in both schizophrenia and Tourette Syndrome patients are observed across sensory modalities as well (Braff et al., 1992; Castellanos et al., 1996; Swerdlow et al., 2001b). Differences in the magnitude of PRA-induced PPI deficits between uni- and cross-modal conditions may reflect psychometric differences between the salience of the acoustic prepulse and light flash prepulse. Nonetheless, these findings suggest that under the present test conditions, unimodal acoustic stimuli may be most sensitive for mechanistic studies of the D3 regulation of PPI.

To date, research on the effects of PRA on PPI in rats has only been conducted in males. PPI is sexually dimorphic in humans (Kumari et al., 2003; Swerdlow et al., 1999)

and in some strains of rats (e.g. Lehmann et al., 1999; Swerdlow et al., 2008a). Studies of estrous cycle phase effects on PPI have had inconsistent results (Adams et al., 2008; Bubenikova et al., 2005; Kinkead et al., 2008; Koch, 1998). Here, we report that PRA-induced PPI deficits can be observed in females as well as males. Furthermore, no significant sex differences in PRA-induced PPI deficits were found. Thus, either male or female rats appear to be suitable for studies of the D3 regulation of PPI, and the preliminary evidence here suggests that estrous phase may not be a confounding factor.

The D3 agonist, pramipexole, appears to be a tool suited for probing the neurobiology of the D3 regulation of sensorimotor gating. The present studies suggest that the PPI-disruptive effects of PRA have a rapid onset and at least a 30 minute duration, and can be separated experimentally from changes in startle magnitude and prepulse-elicited motor reactions. Optimal experimental conditions for studying the PPI-disruptive effects of PRA would appear to include intense startle pulses and unimodal acoustic stimuli, with comparable magnitudes of PRA effects in male and female SD rats, that do not appear to vary across the estrous cycle in females. Added to the findings of past studies of the impact of rat strain (Weber et al., 2008a) and prepulse interval (Swerdlow et al., 2009a), the present parametric analyses provide a fairly broad platform of experimental variables needed for conducting informed studies of the D3 regulation of PPI in rats.

F. Acknowledgements:

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CHAPTER 3:

Stereochemical and neuroanatomical selectivity of pramipexole effects on sensorimotor gating in rats

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A. Abstract:

1. Background: In rats, prepulse inhibition (PPI) of acoustic startle is disrupted by systemic administration of dopaminergic agonists, such as the dopamine D3 receptor (D3R)-preferential agonist pramipexole (PPX). PPX has D3R-active (S) and -inactive (R) stereoisomers. Here, we tested the neuroanatomical and stereochemical selectivity of PPX effects on PPI.

2. Methods: (S)-PRA or (R)-PRA (0, 0.47, 1.42, 4.73 μmol/kg) was injected sc 15 min prior to PPI testing in adult male Sprague Dawley rats. In separate rats, PPX (0, 3, 10 μg/0.5 μl/side, ic) was infused into the nucleus accumbens (NAc), posterior striatum (PS), or olfactory tubercle / Islands of Calleja (ICj) 15 min prior to PPI testing. D3R expression in these brain regions was assessed using quantitative rt-PCR.

3. Results: Systemic administration of PPX stereoisomers demonstrated a dose-dependent effect of (S)-PPX on PPI, while (R)-PPX had no effect on PPI. PPX decreased PPI when infused into the NAc and ICj, but not the PS. Quantitative rt-PCR revealed D3R expression in ICj > NAc > PS.

4. Conclusion: The PPI-reducing effects of PPX are stereospecific for the D3R-active (S)-isomer, and neuroanatomically preferential for the D3R-rich ventral vs. D3R poor dorsal posterior striatum. Both of these findings are consistent with the conclusion that PPX disrupts PPI via stimulation of mesolimbic D3Rs.

B. Introduction:

Prepulse inhibition of acoustic startle (PPI) is the automatic suppression of startle reflex magnitude that occurs when a weak lead stimulus precedes the startling noise. In rats, PPI is disrupted by systemic treatment with dopamine (DA) agonists such as amphetamine and apomorphine; this DA-mediated loss of PPI has been used to model PPI deficits in neuropsychiatric disorders, such as schizophrenia (Braff et al., 1978) and Tourette Syndrome (Castellanos et al., 1996), and to predict the clinical efficacy of antipsychotic compounds (Swerdlow et al., 1994; cf. Swerdlow et al., 2008b). Several studies have assessed the regulation of PPI by specific DA receptor subtypes (Doherty et al., 2008; Peng et al., 1993; Stevenson and Gratton, 2004; Wan and Swerdlow, 1996), and the role of D1, D2 and D3 DA receptors in the regulation of PPI differs substantially across rodent strains and species, as well as across different brain regions. We and others have shown that PPI is disrupted by systemic treatment with D3-preferential agonists such as pramipexole (PPX) and PD128907 (Chang et al., 2010b; Weber et al., 2008a, 2009; Zhang et al., 2007). PPX is among the most D3-selective agonists that are commercially available, with selectivity for D3 over D2 receptors reported to be between 7:1 and 160:1 (Millan et al., 2002; Piercey et al., 1996; Svensson et al., 1994a). We

previously reported the sensitivity of PPX-induced PPI deficits to several experimental parameters, including sex, species, stimulus modality, inter-stimulus interval, and startle-eliciting pulse magnitude, and demonstrated that PPX effects on PPI are dissociable from changes in startle magnitude or prepulse-elicited motor responses (Chang et al., 2010b; Swerdlow et al., 2009a).

Despite its D3-preferential profile, it is unclear precisely how PPX disrupts PPI in rats. For example, a proposed role of D3 autoreceptor stimulation in suppressing presynaptic DA release (Carlsson, 1975; Chen et al., 2009; Diaz et al., 2000) would not easily account for the PPI-disruptive effects of PPX, since PPI is reduced by higher rather than lower levels of forebrain presynaptic DA release (Swerdlow et al., 1990, 2007). PPX has many other biological properties, beyond its effects on D3 receptors, some of which may have clinical utility. For example, PPX exists as both DA-active (S) and DA-inert (R) stereoisomers, both of which display neuroprotective properties in models of DA neurodegenerative disorders, purportedly acting as an antioxidant in neurons (Ferrari-Toninelli et al., 2010; Gu et al., 2004; Joyce et al., 2004; Le et al., 2000; Ramirez et al., 2003). Interestingly, the DA-inactive dexpramipexole has already advanced through early phase clinical trials for neurodegenerative disorders (cf. Cheah and Kiernan, 2010). Here, we first assessed whether D3 activity is necessary for the observed PPX-disruption of PPI, by comparing the effects of (S)-PPX vs. (R)-PPX on this measure.

After establishing that D3-active properties are required for the PPI-disruptive effects of PPX, we next examined whether the PPI-disruptive effects of systemically-administered PPX could be reproduced via intracerebral infusion into discrete forebrain

DA terminal regions. Previous studies have demonstrated that infusion of the D2/D3 agonist quinpirole into the nucleus accumbens (NAc) core and shell regions dose-dependently reduces PPI (Wan et al., 1994). D3 receptors are localized within mesolimbic and limbic regions; highest densities are found in the NAc and Islands of Calleja (ICj) in rats and primates, including humans (Landwehrmeyer et al., 1993; Levesque et al., 1992; Sokoloff et al., 2006). In addition to being implicated in the regulation of PPI, many of these brain regions are also of particular relevance to schizophrenia pathology and/or therapeutics. Here, we assessed changes in PPI in Sprague Dawley rats after infusion of PPX into 3 brain regions: 1) the NAc, based on the prominent role of the NAc in mediating DAergic PPI deficits, and the high D3 receptor expression levels in this brain region; 2) the ICj, based on its reported high levels of D3 receptor expression; and 3) the posterior striatum (PS), a region known to regulate PPI, but which has little or no D3 receptor expression. Levels of D3 expression in these three target regions were confirmed via quantitative RT-PCR of tissue collected from drug-naïve rats.

C. Methods:

1. Experimental Animals: Adult male Sprague-Dawley (n = 54, 225-250 g; Harlan Laboratories, Livermore, CA) rats were housed 2-3 animals per cage, and maintained on a reversed light/dark schedule with water and food available *ad libitum.* Rats were handled within 2 d of arrival. For drug infusion studies, surgery occurred between 7-10 days after arrival. Testing occurred during the dark phase. All experiments

were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, revised 1985) and were approved by the UCSD Animal Subjects Committee (protocol #S01221).

2. Drugs: Pramipexole (PPX), (S)-pramipexole dihydrochloride [(S)-PPX], and (R)-pramipexole dihydrochloride [(R)-PPX] were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Drug doses for systemic PPX stereoisomer salts (0, 0.47, 1.42, 4.73 μmol/kg) are molar equivalents of milligram/kilogram doses used in previous systemic studies with (S)-PPX base (Chang et al., 2010b; Swerdlow et al., 2009a; Weber et al., 2008a, 2009b). Preliminary studies tested the behavioral effects of (S)-PPX base doses compared to molar equivalents of the (S)-PPX salt and found nearly identical effect sizes on PPI and startle magnitude (data not shown). For the PPX stereoisomer studies, drug was injected subcutaneously in a volume of 1 ml/kg body weight 15 min prior to behavioral testing. Drug doses for intracerebral infusions were based on μg/μl base. PPX (0, 3 or 10 μg/0.5 μl saline/side) was infused bilaterally at a rate of 0.5 μl over 42 s. Injectors remained in place for 30 s post-infusion, and then were replaced with a stylet. Rats were placed in the startle chambers after 15 min.

3. Surgery/Histology: Rats received 0.1 ml atropine sulfate (Vedeo, 0.054 mg/ml sc) 15-30 min before being anesthetized with sodium pentobarbital (Abbott, 60.0 mg/kg/ml ip). Fully anesthetized rats were placed in a Kopf stereotaxic instrument (tooth

bar at -3.3 mm). Cannulae (10 mm pieces of 23 ga stainless steel tubing) were lowered 3 mm above specific target sites in the NAc core (AP +1.2, L ±1.7, DV -4.3 (Bregma)), olfactory tubercle containing the ICj (AP +0.7, L ±4.0, DV -5.5 (Bregma)), or PS (AP +0.2, L ±3.5, DV -2.5 (Bregma))(Fig. 3.1A). ICj cannulae were placed at a 10° angle from vertical. Cannulae were anchored to the skull via dental cement and skull screws, and filled with a wire stylet. Rats were monitored daily post-operatively. For i.c. infusions, stylets were removed and 30 ga needles attached to PE 10 tubing were inserted through the cannulae 3 mm beyond their tips.

Histological localization was performed after completion of behavioral testing (Fig. 3.1A). Rats were anesthetized and transcardially perfused with saline followed by 10% buffered formalin. Brains were cryoprotected, then 50 μm coronal sections were cut on a frozen-stage sliding microtome, mounted on glass slides and Nissl-stained. Injector placements were plotted based on the ventral-most extent of the injector tract, and categorized as a "hit" or "miss," blind to behavioral data. One rat from the NAc group was excluded from analysis on this basis.

4. Apparatus: Startle chambers for rats (San Diego Instruments, San Diego, CA, USA) were housed in a sound-attenuated room, and consisted of a Plexiglas cylinder 8.2 cm in diameter resting on a 12.5×25.5 cm Plexiglas frame within a ventilated enclosure. Noise bursts were presented via a speaker mounted 24 cm above the cylinder. A piezoelectric accelerometer mounted below the Plexiglas frame detected and transduced

motion from within the cylinder. Stimulus delivery was controlled by the SR-LAB microcomputer and interface assembly, which also digitized (0-4095), rectified, and recorded stabilimeter readings. One hundred 1-ms readings were collected beginning at stimulus onset. Startle amplitude was defined as the average of the 100 readings.

5. Startle testing procedure: Approximately 7-9 d after shipment arrival for the systemically treated group or 5 d after cannula placement for the intracerebral infusion group, rats were placed in the startle chambers for a 5 min acclimation period with a 70 dB(A) background noise, and then exposed to a total of 17 PULSE-ALONE trials (40 ms − 120 dB(A) noise bursts) that were interspersed with 3 PREPULSE+PULSE trials in which PULSE-ALONE was preceded 100 ms (onset-to-onset) by a 20 ms noise burst, 12 dB above background. Based on this "matching session", rats were assigned to drug dose groups based on average %PPI to ensure similar baseline PPI levels across groups. Starting 2-5 d after the matching session, drug testing began. Inter-test interval was 5-7 d. For both systemic and intracerebral administration studies, drug testing was done on multiple days with PPX dose as a within-subject factor and pseudo-randomized dose order. For stereoisomer studies, (S)-PPX vs. (R)-PPX was a between-subject factor. For drug infusions studies, targeted infusion site was a between-subject factor.

Test sessions began with a 5 min acclimation period with a 70 dB(A) background noise and then a series of trial types presented in pseudorandom order: (1) PULSE-ALONE trials consisting of 40 ms 120 dB(A) noise bursts, (2) PREPULSE+PULSE trials where PULSE-ALONE was preceded 100 ms (onset-to-onset) by a 20-ms noise burst that

was either 5, 10, or 15 dB(A) above background; (3) NOSTIM trials, in which no stimulus was presented but motor activity was measured. The session began with 4 consecutive PULSE-ALONE trials and ended with 3 consecutive PULSE-ALONE trials; between these trials were two blocks, each consisting of eight PULSE-ALONE trials, five 5, 10, and 15 dB(A) PREPULSE+PULSE trials. NOSTIM trials were interspersed between active trials. Average ITI between active trials was 15 s, and NOSTIM trials were not included in the calculation of intertrial intervals. Total session duration was 18.5 min.

6. Quantitative rt-PCR: Two drug- and testing-naïve rats were decapitated and brains rapidly dissected and chilled in ice-cold saline. NAc samples were obtained with a 2 mm diameter punch from 1 mm thick coronal slabs (Bregma +1.2 to +2.2 mm; Fig. 3.1B). The remaining half-moon shaped brain region containing the Islands of Calleja dissected off from this same slab. Posterior striatum was collected with a 2.5 mm diameter punch from the adjacent 1 mm thick coronal slab (Bregma +0.2 to +1.2). All tissue was placed immediately into RNALater (Ambion) and onto dry ice. Samples were stored at -80˚C until RNA extraction with QIAGEN RNeasy Mini Kit. Quantitative rt-PCR was performed by UCSD Center for Aids Research Genomic Core. On-column DNAse digestion was performed with the QIAGEN RNase-free DNase set, and cDNA was made with qScript cDNA SuperMix (Quanta Biosciences). qPCR was conducted on Applied Biosystems 7900HT Fast Real-Time PCR System. Standard curves for DRD3 (Applied Biosystems primer) expression were made with one ICj sample; all samples

were run in duplicate and normalized to GAPDH (Applied Biosystems primer) expression levels. Data are expressed as a fold change from average DRD3 expression in the ICj samples and are an average between duplicates of two brains.

Figure 3.1: Illustration of infusion sites and corresponding areas of rt-PCR analysis. [A]: Diagram of injector placements confirmed by histology post-testing. [B]: Diagram of tissue collection sites for measurement of D3 receptor expression in the brain regions corresponding to PPX infusion sites. Samples were collected bilaterally for each region.
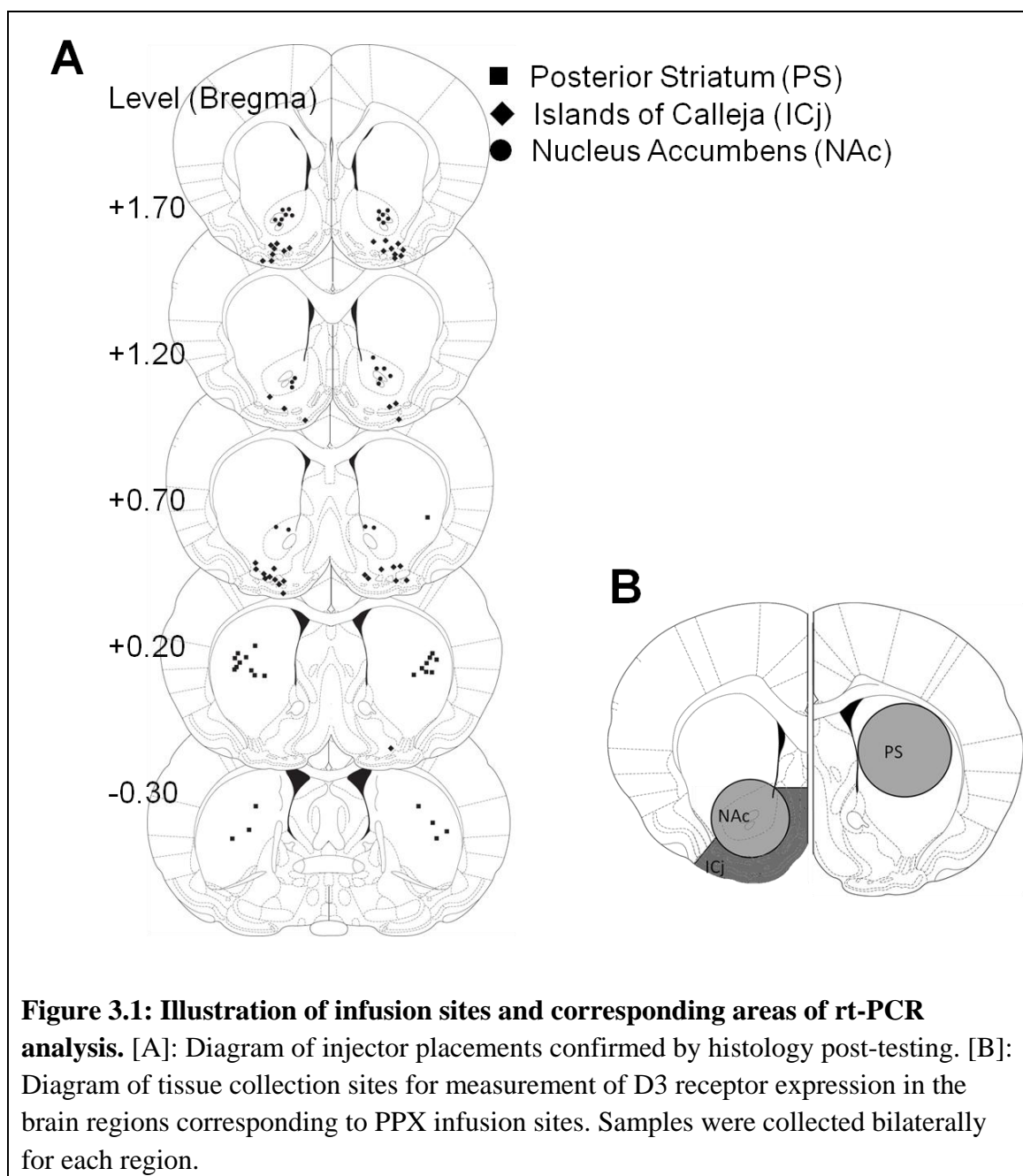
7. Data Analysis: PPI was defined as 100-[(startle amplitude on PREPULSE+PULSE trials/startle amplitude on PULSE-ALONE trials) $\times$ 100], and was analyzed by mixed design ANOVAs. All data were inspected for the presence of "non-responders" defined by a mean startle response to PULSE-ALONE trials of < 10 units (vs. group means of approximately 266 units); none met this criteria. One rat from the ICj infusion group had startle magnitude >5 standard deviations above the group mean and was eliminated from further analyses. Other ANOVAs were used to assess responses on PULSE-ALONE, PREPULSE-ALONE, or NOSTIM trials. Based on differing DRD3 expression levels found with rt-PCR of infusion sites, our *a priori* design was to conduct ANOVAs for PPX effects on PPI within each infusion site group. Where different prepulse intensities were present, data were collapsed across all prepulse intensities, unless otherwise noted based on significant interaction effects. Post-hoc comparisons were conducted using Fisher's protected least significance difference (PLSD). Difference scores for startle magnitude were calculated by averaging the response to PULSE-ALONE across blocks on vehicle test day and subtracting the average PULSE-ALONE response across blocks on high dose (10 µg/0.5 µl PPX) test day. The relationship between startle suppression and PPI effects were tested via comparison of PPI changes after a median split for startle magnitude difference scores. Alpha was set at 0.05.

D. Results:

1. (S)-PPX vs. (R)-PPX effects: Repeated measures ANOVA of %PPI showed significant main effects of stereoisomer (F=14.27, *df* 1,22; p<0.002), PPX dose (F=7.68, *df* 3,66; *p*<0.0003), trial block (F=5.89, *df* 1,22; *p*<0.03), and prepulse intensity (F=108.07, *df* 2,44; *p*<0.0001), and a significant PPX dose × stereoisomer interaction (F=5.91, *df* 3,66; *p*<0.002). There were no other significant two- three- or four-way interactions involving stereoisomer, except for stereoisomer × prepulse intensity (F=4.85, *df* 2,44; *p*<0.02) and stereoisomer × prepulse intensity × PPX dose (F=3.26, *df* 6,132; *p* < 0.006). Both of these interaction effects appeared to reflect a lack of any PPX effect on %PPI in the (R)-PPX treated group. Post-hoc tests revealed that %PPI was significantly reduced by the middle and low doses of (S)-PPX (*p*<0.009, *p*<0.0001, respectively), but were not reduced by any doses of (R)-PPX (Fig 3.2A).

Startle magnitude to PULSE-ALONE trials also demonstrated significant main effects of PPX dose (F=3.48, *df* 3,66; *p*<0.03) and block (F=8.30, *df* 1, 22; *p*<0.009), but interestingly, no effect of stereoisomer (F<1), and no significant interactions. Thus, statistically, both isomers reduce startle magnitude. However, inspection of the data suggests an orderly effect of PPX dose on startle magnitude in the DA-active, but not DA-inactive drug groups (Fig. 3.2B). As in previous studies (Chang et al., 2010b; Swerdlow et al., 2009a; Weber et al., 2008a, 2009b), to understand the relationship between the startle- and PPI-reducing effects of (S)-PPX, difference scores were calculated between startle magnitude after vehicle and after active drug doses, and an ANOVA was conducted with a median split of these difference scores in each

stereoisomer group. The PPI-disruptive effects of PPX did not differ among groups that exhibited high vs. low PPX-induced startle reduction (F<1), nor was there a median split × stereoisomer interaction (F<1).

Figure 3.2: Effects of (S)- vs. (R)-PPX on PPI (A) and startle magnitude (B). [A]: The DA-active (S)-PPX salt induced a dose-dependent reduction in PPI, while the DA-inactive (R)-PPX isomer had no effect on PPI. Drug doses represent molar equivalents of 0.1, 0.3, and 1.0 mg/kg of PPX base previously shown to disrupt PPI (Chang et al., 2010b; Swerdlow et al., 2009a; Weber et al., 2008a, 2009b). [B]: While there was an overall main effect of PPX dose on startle magnitude and no effect of stereoisomer or stereoisomer × PPX dose interaction, the data shown here suggest a dose-dependent effect of (S)-PPX on startle suppression, while the (R)-PPX effect is less orderly. *$p<0.009$, **$p<0.0001$.

2. Quantitative rt-PCR: As expected, highest levels of DRD3 expression were observed in the ICj, and expression levels in other brain regions were calculated as a percentage of ICj expression. NAc expressed 28% and PS expressed 8% as much DRD3 as the ICj.

3. Intracerebral infusion of (S)-PPX: Repeated measures ANOVA of %PPI showed significant main effects of PPX dose in the NAc (F=5.39, *df* 2,13; *p*<0.02) and ICj (F=10.63, *df*2,17; *p*<0.0005) but not the PS (F=2.85, *df* 2,12; NS) (Fig. 3.3A). In all groups, there was a significant main effect of intensity (*p*<0.0001), but no other main effects and no significant interactions. Post-hoc tests revealed that, within the NAc and ICj infusion groups, PPI was significantly decreased at both active PPX doses (*p*'s < 0.05 - 0.01). PPX sensitivity appeared very comparable after infusion into the NAc and ICj; for example, the PPI-reducing effects of the low dose of PPX (3.0 μg) were at least half-maximal (≥ 50% of the 10 μg dose effects) in 44% of ICj group rats, and 43% of NAc group rats. There was no significant effect of PPX infusion on startle magnitude for any site-group (F's = 1.61, < 1 and < 1 for NAc, ICj and PS, respectively; all p's> 0.20), but inspection of the data suggested a trend towards startle magnitude reduction with PPX infusion in the NAc (Fig. 3.3B). Following a similar strategy as that used above for the stereoisomer experiment, difference scores were calculated between startle magnitude after intra-NAc infusion of vehicle and high dose PPX (10 μg). Again, the PPI-disruptive effects of PPX did not differ among groups that exhibited high vs. low PPX-induced startle reduction (F<1).

Figure 3.3: PPX effects on PPI (A) and startle magnitude (B) after intracerebral infusion. A: Significant main effects of PPX infusion on PPI were observed in rats infused in the ICj and NAc, but not the PS. *$p<0.05$; B: There were no significant effects of PPX infusion on startle magnitude, though there appears to be a trend towards dose-dependent startle reduction after PPX infusion in the NAc. NAc=nucleus accumbens, ICj=Islands of Calleja, PS=posterior striatum.
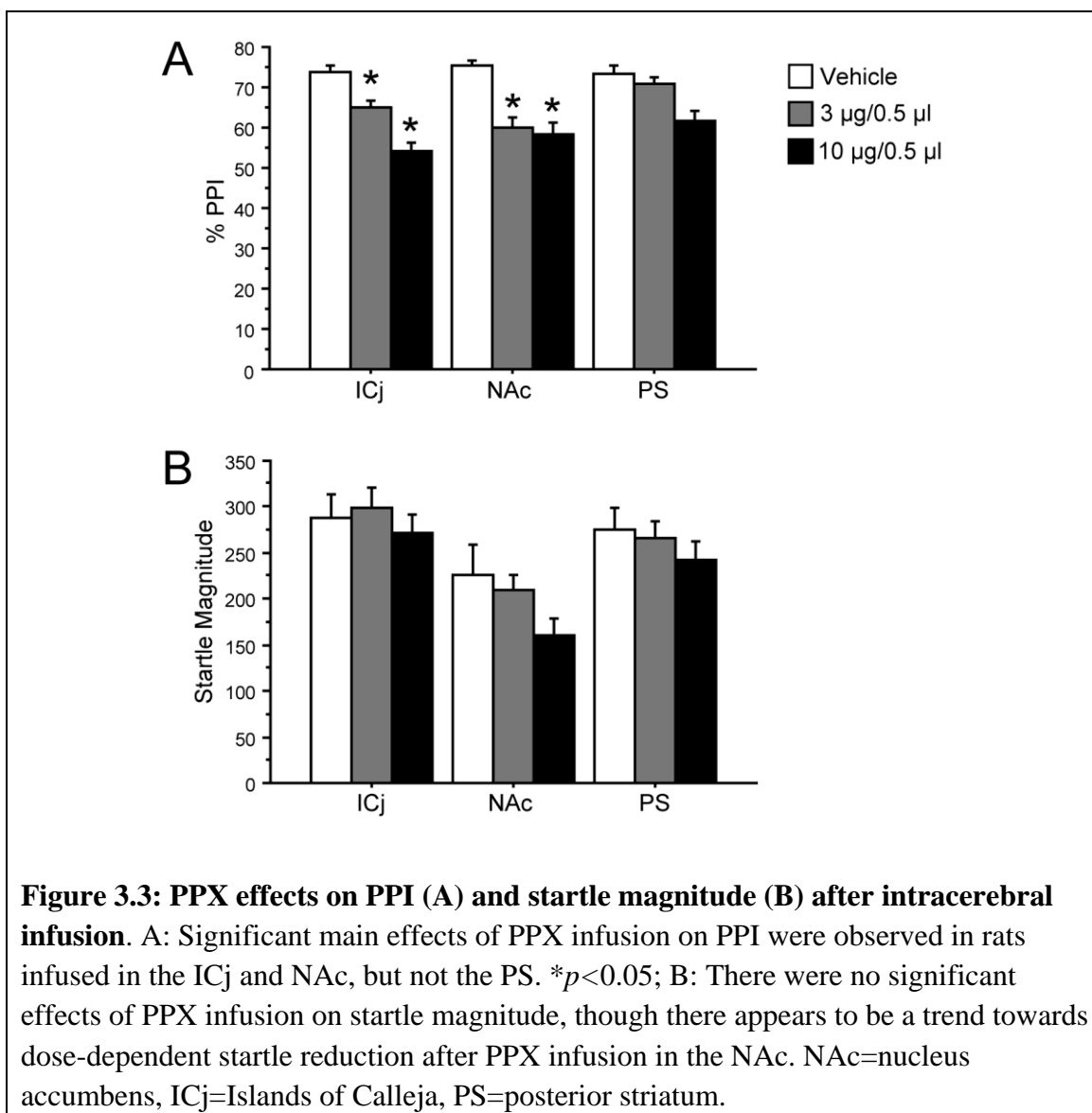
E. Discussion:

Our previous reports of the ability of PPX to disrupt PPI in rats (Weber et al., 2008a, 2009b; Chang et al., 2010b, 2011; Swerdlow et al., 2009a) were based on the assumption that these effects were mediated by interactions between PPX and DA receptors in the forebrain. However, PPX is bioactive in other ways; it has been shown to

be neuroprotective in neurodegenerative models using MPTP, apparently through DA-dependent and DA-independent mechanisms (cf. Albrecht and Buerger, 2009), and the DA-inactive dexpramipexole has demonstrated clinical evidence of neuroprotection in patients with amyotrophic lateral sclerosis (cf. Cheah and Kiernan, 2010). Indeed, in preclinical models, we reported that PPX alters locomotor activity in mice and rats through presumably DA-dependent and DA-independent mechanisms (Chang et al., 2010a, 2011, Richtand et al., 2011). In the present studies, we compared the effects of molar equivalent doses of (S)- and (R)-PPX on startle and PPI in rats. As has been shown previously, (S)-PPX dose-dependently decreased PPI; in contrast, (R)-PPX had no significant effect on PPI, confirming that active DA receptor-stimulating properties of PPX are necessary for its effects on PPI. The dissociation of these isomers was less evident in measures of startle magnitude: both stereoisomers reduced startle magnitude, though the effects of (S)-PPX were more clearly dose-dependent.

Once the stereochemical selectivity of PPX-induced PPI effects was established, we next tested the anatomical selectivity of these effects by infusing PPX directly into brain areas with different levels of DA D3 receptors (DRD3) as measured with quantitative rt-PCR. In keeping with what has been shown through radiolabeling, *in situ* hybridization, and immunohistochemistry studies (Bouthenet et al., 1991; Diaz et al., 1995, 2000; Herroelen et al., 1994; Schwartz et al., 2000; Suzuki et al., 1998), we found the highest levels of DRD3 in the ICj -- roughly 4 times higher than in the NAc, and 12-13 times higher than the PS. Based on the differential DRD3 expression levels, as well as varying degrees of involvement of these regions in startle circuitry (Swerdlow et al.,

2000, 2001a), we hypothesized that: 1) the PPI-disruptive effects of PPX after systemic drug infusion could be reproduced by direct infusion into forebrain DA terminal regions that regulate PPI; and 2) the different infusion sites would demonstrate different sensitivities to the PPI-disruptive effects of PPX. Indeed, direct infusion of PPX was sufficient to significantly reduce PPI. Moreover, regions with higher DRD3 expression -- the ICj and NAc -- appeared to be sensitive to these PPX effects, while PPX infusion into the PS did not disrupt PPI. This differential sensitivity to the PPI-disruptive effects of PPX might certainly reflect a more prominent role of the ventral striatum vs. the PS in the regulation of PPI; on the other hand, the PS also regulates PPI, since lesions of the PS and pharmacological manipulations of its efferent projections are known to significantly disrupt PPI in rats (Kodsi and Swerdlow, 1995) and mice (Takahashi et al., 2007).

Other nonselective D2/D3 agonists can induce PPI deficits with localized infusion in the brain, with the strongest effect being observed in regions of the NAC (e.g. Wan et al., 1994). To the best of our knowledge, the present data provide the first suggestion for a role of the ICj in the DAergic regulation of PPI. Interestingly, despite the fact that the ICj has a 4-fold higher level of DRD3 expression compared to the NAc, the PPI-disruptive effects of the D3 agonist, PPX, were not greater - and, in fact, arithmetically diminished - compared to the NAc. Thus, it appears that the relative levels of DRD3 expression cannot fully account for the sensitivity to the PPI-disruptive effects after intracerebral infusion of PPX.

The present findings demonstrate that the PPI-disruptive effects of PPX are selective to its D3R-active S-enantiomer, and are mediated predominantly within the

D3R-rich ventral striatum more so than the D3R-poor dorsal posterior striatum. These findings do not, however, definitively resolve whether these PPX effects are mediated by its predominant D3-stimulatory properties, vs. its activity at other D2-family receptors. We have reported that, compared to selective D2 agonist sumanirole, the mixed D1/D2 agonist apomorphine, and even the relatively D3-favoring agonists 7-OH-DPAT and PD128907, the PPI-disruptive effects of PPX are relatively insensitive to "rescue" by selective D2 receptor blockade (Breier et al., 2011; Weber et al., 2009b, 2010b). However, a clear demonstration that the PPI-disruptive effects of PPX reflect its action at D3 receptors has been hindered by the relative paucity of highly selective and potent D3 receptors antagonists, and by apparent species differences in the DA receptor-specific regulation of PPI (Chang et al., 2010a). Resolving this issue will become increasingly important in understanding the mechanisms of action of PPX, which is being advanced in humans as a putative pro-cognitive agent, with potential applications in the treatment of schizophrenia and other neuropsychiatric disorders (Ersche et al., 2011; Swerdlow, 2011).

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CHAPTER 4:

Technical note:

Pharmacological parsing of D2- vs. D3-mediated regulation of sensorimotor gating

in rats

Wei-li Chang, Michelle R. Breier, Martin Weber, Neal R. Swerdlow

A. Abstract:

1. Background: Prepulse inhibition of acoustic startle (PPI) is the automatic suppression of startle reflex magnitude that occurs when a weak lead stimulus precedes the startling noise. Disruption of PPI in rats by systemic treatment with dopamine (DA) agonists is used to model PPI deficits in neuropsychiatric disorders, such as schizophrenia, and to predict the clinical efficacy of antipsychotic candidates. The role of DA receptor subtypes in the regulation of PPI may ultimately provide important clues for rational drug design, but to date remains poorly understood: for example, the PPI-regulatory roles of D1, D2 and D3 receptors differ substantially across rodent strains and species, as well as across different brain regions. In rats, attempts to dissociate the D2- vs. D3-mediated regulation of PPI are complicated by the significant structural and distributional homology of these receptors, and a lack of D3-selective pharmacologic or molecular tools. Here, we compared the relative sensitivity of the PPI-disruptive effects of different D3-preferential agonists to blockade by a selective D2 antagonist, L741626.

2. Methods: Acoustic startle and PPI were assessed in male Sprague Dawley rats after pretreatment with differing doses of L741626 and treatment with the highly selective D2 agonist, sumanirole (SUM), or the D3-preferential agonists, pramipexole (PPX), PD128907 or 7-OH-DPAT.

3. Results: The PPI-disruptive effects of the D2-selective agonist, SUM, were highly sensitive to L741626, with statistically significant blockade achieved by L741626 doses of 0.1 mg/kg, consistent with its potent D2-blocking abilities. Comparable degrees of PPI disruption produced by D3-preferential agonists, PPX, PD128907 and 7-OH-DPAT were significantly opposed only by higher doses of L741626: 3.0, 1.0 and 1.0 mg/kg, respectively.

4. Conclusion: Compared to the PPI-disruptive effects of SUM, the same degrees of PPI disruption produced by 3 different D3-preferential agonists were 10 - 30 times less sensitive to D2 blockade. Among these D3 agonists, the PPI-disruptive effects of PPX appear to be least dependent on the availability of D2 receptors. Given our limits to precisely dissect D2- vs. D3-substrates of PPI in rats, PPX appears to offer advantages of specificity over other available D3 agonists.

B. Introduction:

The dopamine (DA) D3 receptor is a promising target for novel neuropsychiatric pharmacotherapies. One behavioral model used heavily for its preclinical predictive validity of DAergic agents is prepulse inhibition (PPI) of acoustic startle reflex. PPI is an

operational measure of sensorimotor gating, that is deficient in several brain disorders, including schizophrenia (Braff and Freedman, 2002). PPI deficits can be induced experimentally in rats by administration of mixed DA receptor agonists. Recently, compounds have become available that display selectivity of D3 (vs. D2) receptors, and which may exhibit novel clinical profiles. For example, the preferential D3R agonist pramipexole (PPX) has a D3:D2 binding preference ranging from 7:1 to 160:1 *in vitro* (Millan et al., 2002; Piercey et al., 1996; Svensson et al., 1994a)*,* and along with the D3-preferential agonist ropinirole, has both novel therapeutic value in treating specific movement disorders, and a novel liability profile in inducing specific behavioral syndromes characterized by high hedonic or novelty valence (cf. Ahlskog, 2011). Both PPX and ropinirole, along with other D3-preferential agonists 7-OH-DPAT and PD128907, have been shown to have PPI-disruptive effects (Caine et al., 1995; Chang et al., 2010b; Swerdlow et al., 2009a; Varty et al., 1998; Weber et al., 2008a, 2009b; Zhang et al., 2007). 7-OH-DPAT has an *in vitro* D3:D2 binding preference ranging from 5:1 to 178:1 (Levant, 1997; Sautel et al., 1995), and PD128907 has a binding preference ranging from 18:1 to 53:1 (Pugsley et al., 1995, Sautel et al., 1995). Studies using DA receptor knockout mice have had difficulty in discerning the contribution of D2 vs. D3 receptor activation in the behavioral effects of these D3-preferential agonists (Ahlenius and Salmi, 1994; Bancroft et al, 1998; Boulay et al, 1999a, 1999b; Daly and Waddington, 1993; Levant et al, 1996; Pugsley et al, 1995; Siuciak and Fujiwara, 2004; Xu et al, 1999).

The current study aims to use a selective D2 antagonist, L741626 (Millan et al., 2000c), to parse the D2- vs. non-D2 contributions to the PPI-disruptive effects of PD128907, 7-OH-DPAT, and PPX. Each agonist will be tested for its relative sensitivity to blockade of PPI effects by L741626, and these values will be compared to sensitivity of the D2-preferential agonist sumanirole (SUM), which has also been shown to have PPI-disruptive effects (Weber et al., 2010b). The agonist exhibiting least sensitivity to blockade by L741626 will then be studied in subsequent behavioral, biochemical and molecular analyses to understand the D3 regulation of PPI in rodents.

C. Methods:

1. Experimental Animals: Adult male Sprague-Dawley (n = 120, 225-250 g; Harlan Laboratories, Livermore, CA) rats were housed 2-3 animals per cage, and maintained on a reversed light/dark schedule with water and food available *ad libitum.* Rats were handled within 2 d of arrival and acclimated for 7 d before behavioral testing, which occurred during the dark phase. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, revised 1985) and were approved by the UCSD Animal Subjects Committee (protocol #S01221).

2. Drugs: All drugs were administered sc. in a volume of 1 ml/kg body weight. For other information, see Table 4.1.

Table 4.1: Drug information

Drug	Vendor	Class	Vehicle	Dose (mg/kg)	Pretreatment Time
L741626	Tocris	Antagonist	0.1% Lactic Acid in Sterile Water	0, 0.03, 0.1, 0.3, 0.6, 1.0, 3.0	30 min before agonist
Sumanirole (SUM)	NIMH	Agonist	Saline	0, 3.0	15 min before PPI testing
PD128907	Tocris	Agonist	Sterile Water	0, 0.2	5 min before PPI testing
7-OH-DPAT	Sigma	Agonist	Saline	0, 0.2	5 min before PPI testing
Pramipexole (PPX)	TRC	Agonist	saline	0, 1.0	15 min before PPI testing

3. PPI Testing: Approximately 7-9 d after shipment arrival, rats were exposed to a short "matching" startle session. They were placed in the startle chambers for a 5 min acclimation period with a 70 dB(A) background noise, and then exposed to a total of 17 PULSE-ALONE trials (40 ms − 120 dB(A) noise bursts) that were interspersed with 3 PREPULSE+PULSE trials in which PULSE-ALONE was preceded 100 ms (onset-to-onset) by a 20 ms noise burst, 12 dB above background. Rats were assigned to drug dose groups based on average %PPI from the matching session to ensure similar baseline PPI levels between groups. Starting 2-5 d after the matching session, drug testing began. Inter-test interval was 4-7 d. Drug testing was done on multiple days with agonist as a within-subjects factor and antagonist dose as a between-subjects factor. Test sessions began with a 5 min acclimation period with a 70 dB(A) background noise. Active trials were presented in pseudorandom order and included: (1) PULSE-ALONE (40 ms − 120

dB(A) noise burst); (2-4) PREPULSE+PULSE (PULSE-ALONE preceded 100 ms (onset-to-onset) by a 20 ms noise burst either 5, 10, or 15 dB above background). Interspersed between active trials were NOSTIM trials in which no stimulus was presented but activity was recorded. Average ITI between active trials was 15 s. PPI test session was approximately 30.5 min in duration (divided into 3 test blocks), except for L741626 vs. PPX testing, which had an 18.5 min test duration (divided into 2 test blocks). Test blocks in all sessions were the same length.

D. Results:

1. L741626 vs. Sumanirole (SUM): A range of doses of the D2-preferential antagonist L741626 (0, 0.03, 0.1, 0.3 mg/kg) were administered before injection of the D2-preferential agonist SUM (0, 3.0 mg/kg) at a dose known to cause PPI disruption (Weber et al., 2010b) in order to test for relative sensitivity of SUM to blockade of PPI effects. Repeated measures ANOVA of %PPI revealed significant main effects of SUM (F=28.03, *df* 1,44; *p*<0.0001), prepulse intensity (F=270.78, *df* 2,88; *p*<0.0001), and testing blocks (F=15.78, *df* 2,88; *p*<0.0001). There was no main effect of L741626 dose (F=1.32, *df* 3,44; NS), but a significant interaction of L741626 × SUM was detected (F=3.77, *df* 3,44; *p*<0.02) with no significant three-way interactions. Data were collapsed across prepulse intensities for subsequent analyses. Post-hoc testing with Fisher's PLSD indicated significantly higher PPI levels in SUM-treated rats that were pretreated with 0.1 and 0.3 mg/kg L741626, compared to those pretreated with vehicle (*p*<0.05)(Fig. 4.1).

Figure 4.1: Effects of L741626 pretreatment on SUM-induced PPI deficits.
Animals received L741626 pretreatment (0, 0.03, 0.1, 0.3 mg/kg) 30 min prior to
SUM (0, 3.0 mg/kg) injection and PPI testing 15 min later. * indicates lowest dose of
L741626 that significant blocks PPI effects of SUM. *N*=48
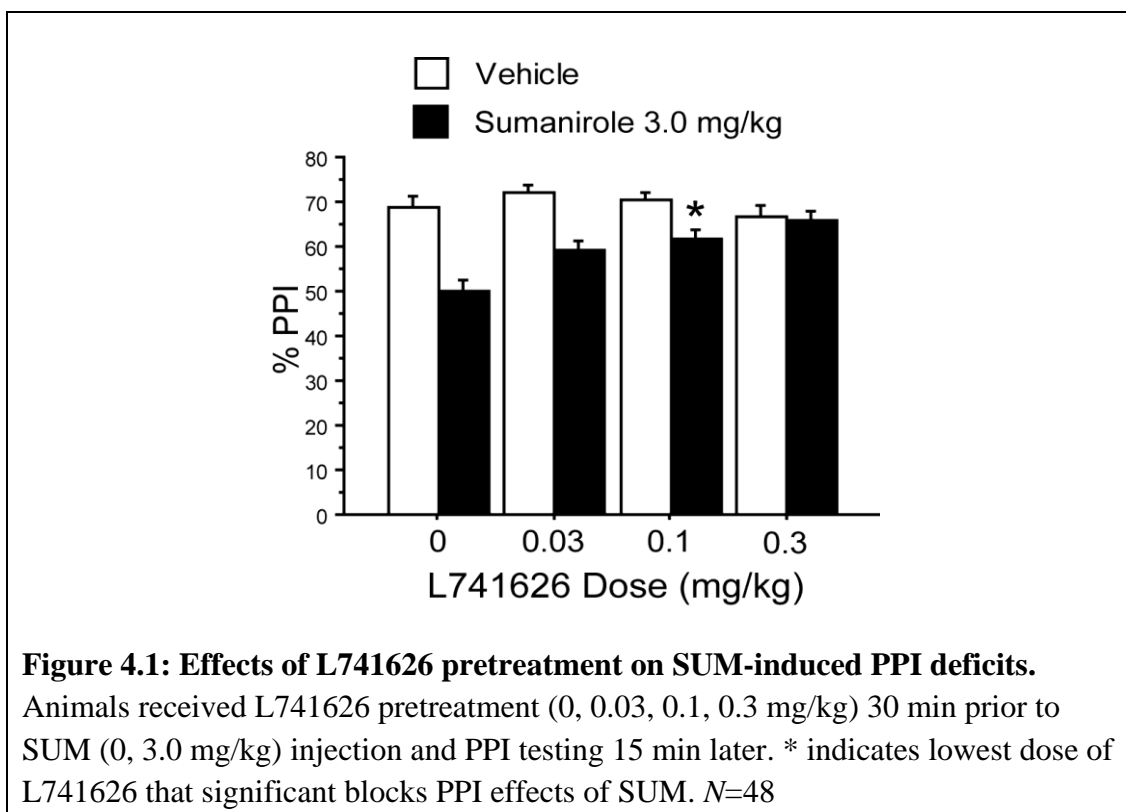
2. L741626 vs. PD128907 and 7-OH-DPAT: A similar design was used to test
sensitivity of the D3-preferential agonists PD128907 (0, 0.2 mg/kg) and 7-OH-DPAT to
pretreatment with L741626 (0, 1.0 mg/kg), using a doses of these D3 agonists that
produced a comparable level of PPI disruption to that produced by SUM 3.0 mg/kg. For
PD128907, ANOVA revealed significant main effects of PD128907 (F=27.44, *df* 1,14;
p<0.0002) and intensity (F=50.43, *df* 2,28; *p*<0.0001). The main effect of L741626 dose
approached significance (F=4.17, *df* 1,14; *p*=0.061), and there was a significant L741626
× PD128907 interaction (F=12.78, *df* 1,14; *p*<0.004). No other meaningful 2- or 3-way
interactions were found and data were collapsed across intensities. Post-hoc tests showed
that PPI in PD128907-treated rats was significantly increased by 1.0 mg/kg L741626

(Fig. 4.2). For 7-OH-DPAT, ANOVA revealed significant main effects of 7-OH-DPAT (F=50.91, *df* 1,22; *p*<0.0001), L741626 (F=13.32, *df* 1,22; *p*<0.002), and intensity (F=32.68, *df* 2,44; *p*<0.0001), and a L741626 × 7-OH-DPAT interaction (F=12.13, *df* 1,22; *p*<0.003). No other meaningful 2- or 3-way interactions were found and data were collapsed across intensities. Post-hoc tests indicated that, as it did with PD128907, L741626 1.0 mg/kg significantly increased PPI in 7-OH-DPAT-treated rats (*p*<0.004) (Fig. 4.2).

3. L741626 vs. Pramipexole (PPX). Similar to the above tests with SUM, PD128907 and 7-OH-DPAT, we next tested the sensitivity of the PPI-disruptive effects of the D3-preferential agonist, PPX (0, 1.0 mg/kg), to blockade by L741626 (0, 1.0, 3.0 mg/kg). ANOVA demonstrated significant main effects of PPX (F=17.14, *df* 1,21; *p*<0.0006), L741626 (F=4.12, *df* 2,21; *p*<0.04), and intensity (F=44.93, *df* 2,42; *p*<0.0001), and only a trend towards a significant L741626 × PPX interaction (*p*=0.067). However, based on our *a priori* hypothesis that higher doses of L741626 might significantly block PPX-induced PPI effects, a separate ANOVA of PPI in PPX-treated animals was conducted, and revealed a significant effect of L741626 (F=4.49, *df* 2,21; *p*<0.03), which was found in post-hoc testing to be due to a significant difference between vehicle and L741626 3.0 mg/kg groups (*p*<0.03) (Fig. 4.2). Thus among all the D3-preferential agonists tested with this method, the PPI-disruptive effects of PPX appeared to be the least sensitive (approximately by 3-fold, compared to PD128907 and 7-OH-DPAT) to blockade by the D2-preferential antagonist L741626. These effects of

PPX were also roughly 30 times less sensitive to L741626 than was the D2-preferential agonist SUM.

Figure 4.2: Effects of L741626 pretreatment against PPI deficits induced by D3 agonists. L741626 was injected 30 min prior to either PD128907 (0, 0.2 mg/kg), 7-OH-DPAT (0, 0.2 mg/kg), or pramipexole (PPX) (0, 1.0 mg/kg). * indicates the lowest dose of L741626 that significantly blocks effects of the D3 agonists on PPI ($p<0.05$). *N=72*
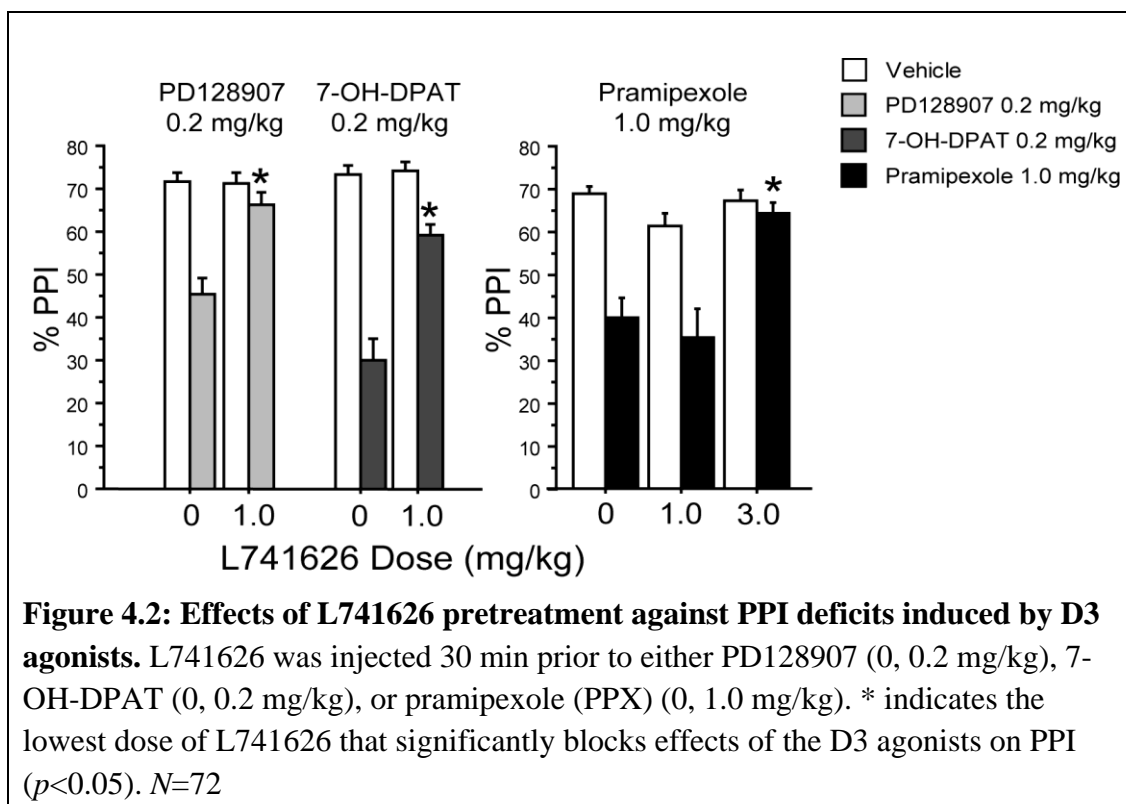
E. Discussion:

The findings of relative insensitivity PPX vs. SUM to the ability of L741626 to prevent the disruption of PPI is in keeping with previous findings (Weber et al., 2008a, 2009b, 2010b). Importantly, the present studies suggest that the PPI-disruptive effects of PPX also remain intact after blockade of D2 receptors at levels that significantly prevent the PPI-disruptive effects of other D3-preferential agonists, PD128907 and 7-OH-DPAT.

This observation suggests that PPX-disrupted PPI may be less dependent on D2 receptors, compared to the PPI-disruptive effects of these other D3-preferential agonists, and that PPX may thus offer advantages as a tool for understanding the regulation of PPI by D3 receptors in rodents.

Certainly, confirmation for a role of D3 receptors in these effects of PPX would be facilitated by the availability of potent, selective and behaviorally active D3 receptor antagonists. Previously, selective blockade of D3 receptors in awake, behaving rats has been complex due to low bioactivity and dose-limiting adverse reactions (Audinot et al., 1998; Millan et al., 2000a, 2000b, 2008b). In our experience with two D3-preferential antagonists ABT 925 (Graff-Guerrero et al., 2010) and SB277011, neither drug exhibited any evidence of bioactivity in Sprague Dawley rats across many different behavioral measures and at doses up to the limits of solubility (unpublished observation). The ineffectiveness of the D3 receptor antagonist, UH-232, to oppose the PPI-disruptive effects of 7-OH-DPAT was previously reported by this laboratory (Caine et al., 1995). Preliminary studies from our group using the D3-selective antagonist, U99194 (Breier et al., 2011) suggest that it can significantly oppose the PPI-disruptive effects of PPX, and ongoing studies are assessing the receptor selectivity of this effect via studies of U99194 effects on SUM-disrupted PPI. U99194 has previously been shown to inhibit behavioral changes elicited by D3-preferential agonists such as yawning (Collins et al., 2005) and hypolocomotion (Carr et al., 2002; Millan et al., 2004b).

In summary, the D2-selective antagonist, L741626 significantly reverses the PPI-disruptive effects of the D2-selective agonist, SUM, and the D3-preferential agonists,

PD128907, 7-OH-DPAT and PPX. However, the sensitivity of these DA agonists to L741626 in this measure vary widely, with PPX being approximately 30 times less sensitive to L741626 than was SUM, and at least 3 times less sensitive to L741626 than were either PD128907 and 7-OH-DPAT. This differential L741626 sensitivity was evident using doses of each DA agonist that produced a comparable degree of disruption of PPI. While we await confirmation of these findings using D3-seletive antagonists, the present results provide a basis for choosing PPX for future behavioral, biochemical and molecular analyses designed to understand the neural mechanisms underlying the D3 regulation of PPI in rodents.

CHAPTER 5:

Using prepulse inhibition to detect functional D3 receptor antagonism:

Effects of WC10 and WC44

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Robert R. Luedtke, Robert H. Mach, Neal R. Swerdlow

A. Abstract:

Prepulse inhibition of startle (PPI) is an operational measure of sensorimotor gating that is impaired in schizophrenia. Treatment with mixed dopamine D2/D3 antagonists diminishes schizophrenia symptoms, and opposes dopamine agonist-induced PPI deficits in rats. There are reasons to believe that functional D3 receptor antagonists might offer more favorable therapeutic profiles compared to current antipsychotics. However, D3-related drug discovery is hampered by the absence of assays sensitive to D3-mediated (antipsychotic) properties *in vivo*. Here, we characterized two putative D3-active compounds - WC10 and WC44 - in a PPI-based screening assay, comparing the sensitivity of test compounds to oppose PPI deficits induced by the mixed D1/D2-like agonist apomorphine vs. the preferential D3 agonist pramipexole in rats. WC10, WC44 (0, 1, 3, 10 mg/kg, each), and the preferential D2 antagonist L741,626 (0, 1 mg/kg) were studied, in combination with apomorphine (0, 0.5 mg/kg), or pramipexole (0, 1 mg/kg). L741,626 prevented apomorphine-, but not pramipexole-induced PPI deficits. WC10, but

not WC44, prevented apomorphine-induced PPI deficits; both compounds opposed pramipexole-induced PPI deficits, suggesting functional D3 and D1/D2 antagonist profiles for WC10, and functional D3 receptor antagonism for WC44. This assay may be valuable for detecting predominantly D3 vs. D2 receptor-linked mechanisms of action *in vivo*.

B. Introduction:

It is suggested that some of the therapeutic effects of D2/D3 antagonist antipsychotics are mediated via blockade of D3 receptors, while their extrapyramidal side effects are due primarily to D2 receptor antagonism (Sokoloff et al., 1990). One basis for this hypothesis is that, compared to D2 receptors, D3 receptors are localized primarily in limbic and mesolimbic regions, while D2 receptors are distributed throughout the striatum. Very high densities of D3 receptors are found in the nucleus accumbens (NAC) of both rats and primates (cf. Sokoloff et al., 2006), and this brain region is implicated in both the pathophysiology of schizophrenia and the therapeutic mechanisms of antipsychotics (cf. Gurevich et al., 1997). Preferential antagonists or partial agonists for D3 receptors might thus offer therapeutic advantages over current antipsychotics.

Developing compounds with a D3 preferential *in vivo* profile, however, is complicated by the high sequence homology of D3 and D2 receptors (cf. Luedtke and Mach, 2003). Further, such compounds may be subject to functional selectivity, i.e. may act as agonist, partial agonist, or antagonist on a given receptor, depending on tissue

types, availability of certain G-proteins and the intracellular machinery linked to these receptors/G-proteins (cf. Mailman, 2007). This raises the possibility that DA compounds that have been characterized as agonists or antagonists for specific DA receptor subtypes *in vitro* might nonetheless have different functional properties *in vivo*. Thus, while the D3 receptor is a promising target for antipsychotic development, and *in vitro* studies have been very valuable in identifying compounds that preferentially bind to D3 receptors (cf. Joyce and Millan, 2005), better models are needed, with 1) predictive validity for antipsychotic-like function; 2) sensitivity for the detection of a predominant D3 related (vs. D1/D2 related) mechanism of action *in vivo*.

One valuable, translational and predictive model for antipsychotic function is prepulse inhibition (PPI). PPI is an operational measure of sensorimotor gating, defined by the reduction in startle magnitude following a weak prestimulus. PPI is impaired in unmedicated schizophrenia patients (Braff et al., 1978; Swerdlow et al., 2006b) as well as their unaffected first-degree relatives (Cadenhead et al., 2000; Kumari et al., 2005), and a recent study linked polymorphisms of the DA D3 receptor to levels of PPI in healthy controls (Roussos et al., 2008a). In rats, PPI deficits are induced by DA agonists such as the direct D1/D2-like agonist apomorphine (APO) and the indirect DA agonist amphetamine (Swerdlow et al., 1986; Mansbach et al., 1988). PPI is potently regulated by brain regions rich in D3 receptors, in particular the NAC (Swerdlow et al., 1986; cf. Swerdlow et al., 2008b), and D3-preferential agonists such as 7-OH-DPAT, ropinirole and quinelorane disrupt PPI in rats and humans (Caine et al., 1995; Giakoumaki et al., 2007; Swerdlow et al., 1998a; Varty and Higgins, 1998). However, the limited D3 vs. D2

binding preference of these compounds makes it difficult to assess the relative contribution of D3 vs. D2 receptor activation to these effects.

This study assessed the ability of the rat PPI model to detect compounds with predominant functional D3 antagonism *in vivo*, based on a *greater sensitivity* to prevent PPI deficits induced by a preferential D3 agonist (e.g. pramipexole (PRA)) compared to a non-selective D1/D2 agonist (e.g. APO). This strategy is based on the contrasting binding affinities of PRA and APO to DA receptor subtypes. Millan et al. (2002) reported that the binding affinity of PRA to the D3 receptor (hD3) was 90-fold higher relative to the short isoform of the receptor (D2S), 160-fold higher relative to the long isoform (D2L), and more than 10,000-fold relative to the D1 receptor (hD1). In contrast, the binding affinity of APO to the hD3 receptor was only 1.35-fold higher than to the D2S, 3-fold higher than to the D2L, and only 14-fold higher than to the hD1 receptor, confirming the non-selectiveness of APO to these DA receptor subtypes. These data indicate that a positive finding with the *in vivo* assay used here could potentially provide functional evidence of greater D3 than D1/D2 selectivity, and predict potentially novel clinical profiles. Another important basis for this *in vivo* assay is the finding that the selective D2 antagonist L741,626 is more sensitive in its ability to prevent PPI deficits caused by APO than those caused by PRA (Weber et al., 2008a). This suggests a critical role of D2 receptor activation in the PPI-disruptive effects of APO, but not PRA. A similar approach was used by Zhang et al. (2007) to demonstrate that SB-277011-A and A-69110 - two putative D3 receptor antagonists - prevented PPI deficits induced by the preferential D3 agonist PD128907, but *not* those induced by APO.

WC10 and WC44 are phenylpiperazine derivates (Fig. 5.1) that have been characterized in forskolin-stimulated adenylate cyclase activity assays *in vitro*, using cell systems expressing either D3- or D2 receptors (Chu et al., 2005). Using these and binding assays, WC10 has been characterized as an antagonist/weak partial agonist with a D3:D2 binding ratio of 43, and WC44 as a full D3 agonist (but see below) with a D3:D2 binding ratio of 23 (Chu et al., 2005). In the present studies, the potential preclinical antipsychotic-like profile of WC44 was evaluated in measures of PPI deficits induced by APO and PRA based on 1) initial experiments showing that WC44 did <u>not</u> have a D3 agonist-like profile *in vivo* (Fig. 5.3), 2) the structural similarities between WC44 and the D3 antagonist/weak partial agonist WC10 (Fig. 5.1), and 3) similar *in vivo* effects of WC10 and WC44 in a study by Kumar et al. (2009).

Figure 5.1: Structures of the two phenylpiperazine derivates WC44 (A), and WC10 (B).
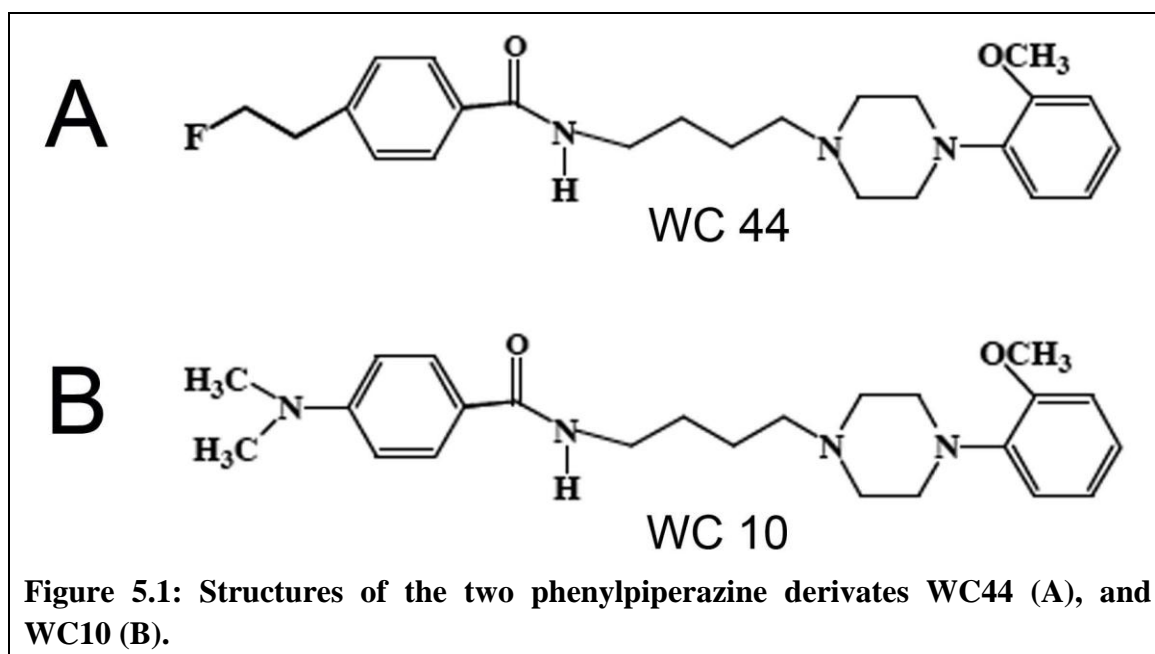
C. Methods:

1. Experimental animals: Adult male SD rats (n = 184; 225-250 g; Harlan Laboratories, Livermore, CA) were housed in groups of 2-3 animals per cage, and maintained on a reversed light/dark schedule with water and food available *ad libitum*. Rats were handled within 2 d of arrival. Testing occurred during the dark phase. All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and were approved by the UCSD Animal Subjects Committee (protocol #S01221). All behavioral testing was completed in a laboratory that is free of all proprietary interests in WC10 or WC44 (MW, WLC, PEP, NRS).

2. Drugs: Apomorphine HCl hemihydrate was purchased from Sigma-Aldrich (St. Louis, MO, USA), PRA from Toronto Research Chemicals (North York, On, Canada), and L741,626 from Tocris (Ellisville, MO, USA). WC10 and WC44 were synthesized by J.P. Durbin according to published methods (structures 12b and 12i, respectively, in Chu et al., 2005). Drug doses are based on mg/kg of salts. APO, PRA, and L741,626 were administered subcutaneously (sc). WC10 and WC44 were administered intra-peritoneally (ip). PRA (0, 1.0 mg/kg) was dissolved in saline, and APO (0, 0.5 mg/kg) was dissolved in 0.01 % ascorbate/saline. L741,626 (water vehicle, or 1 mg/kg) was dissolved in 0.05% lactic acid/water (w/v) and pH was adjusted to ≥5 using NaOH. WC10 (5% DMSO/water (v/v) vehicle, 1, 3, or 10 mg/kg) was dissolved in

DMSO; water and a few drops of 1N HCl were added to achieve a final 5%DMSO/water (v/v) (+HCl) solution. WC44 (5% DMSO/water (v/v) vehicle, 1, 3, or 10 mg/kg) was dissolved in DMSO followed by the addition of water to achieve a final 5%DMSO/water (v/v) solution. All injection volumes were 1 ml/kg.

3. Apparatus: Startle chambers for rats (San Diego Instruments, San Diego, CA, USA) were housed in a sound-attenuated room, and consisted of a Plexiglas cylinder 8.2 cm in diameter resting on a 12.5×25.5 cm Plexiglas frame within a ventilated enclosure. Noise bursts were presented via a speaker mounted 24 cm above the cylinder. A piezoelectric accelerometer mounted below the Plexiglas frame detected and transduced motion from within the cylinder. Stimulus delivery was controlled by the SR-LAB microcomputer and interface assembly, which also digitized (0-4095), rectified, and recorded stabilimeter readings. One hundred 1-ms readings were collected beginning at stimulus onset. Startle amplitude was defined as the average of the 100 readings.

4. Startle testing procedure: Approximately 7 days after shipment arrival, rats were exposed to a short "matching" startle session. They were placed in the startle chambers for a 5 min acclimation period with a 70 dB(A) background noise, and then exposed to a total of 17 P-ALONE trials (40 ms - 120 dB(A) noise bursts) that were interspersed with 3 PREPULSE+PULSE trials in which P-ALONE was preceded 100 ms (onset-to-onset) by a 20 ms noise burst, 12 dB above background. Rats were assigned to

drug dose groups based on average %PPI from the matching session to ensure similar baseline PPI levels between groups. Starting 2-5 d after the matching session, drug testing began. One study assessed the effects of WC44 alone, in a one-day study with the WC44 dose (0, 1, 3, 10 mg/kg; i.p.) as the between-subjects factor. Tests of L741,626 (0, 1 mg/kg), WC10 (0, 1, 3, 10 mg/kg), or WC44 (0, 1, 3, 10 mg/kg) vs. APO (0 or 0.5 mg/kg) or PRA (0 or 1 mg/kg) were two day-studies and had a mixed-model, balanced dose-order (vehicle vs. active dose of the agonist) design with L741,626, WC10, or WC44 pretreatments as the between factor and APO or PRA treatments as the within factor.

In the study of WC44 alone, rats were treated with WC44 (0, 1, 3, 10 mg/kg) and placed into the startle chambers 10 min thereafter. Experiments testing L741,626 vs. APO or PRA used a pretreatment time for L741,626 relative to APO or PRA of 30 min. Experiments testing WC10, WC44 vs. APO or PRA used a pretreatment time of 10 min relative to APO treatment, or 5 min relative to PRA treatment. Rats were placed into the startle chambers immediately after APO treatment or 15 min after PRA treatment. Pretreatment intervals were based on pharmacological data from Kumar et al. (2009), and pilot experiments from our laboratory.

The PPI test session began by placing the rats in the startle chambers followed by a 5 min acclimation period with a 70 dB(A) background noise. Rats were then exposed to a series of trial types, which were presented in pseudorandom order. Interspersed between these active trial types were trials in which no stimulus was presented, but cage displacement was measured (NOSTIM trials). The session consisted of the following trial

types: (1) P-ALONE; (2-4) P-ALONE preceded 100 ms (onset-to-onset) by a PREPULSE (PP) consisting of a 20 ms noise burst of either 5, 10, or 15 dB above background (PP5+PULSE, PP10+PULSE, or PP15+PULSE trials, respectively). The session began with 4 consecutive P-ALONE trials and ended with 3 consecutive P-ALONE trials; between these trials were three blocks, each consisting of 8 P-ALONE trials, and 5 trials of each prepulse + pulse combination. Trial blocks were used to assess the time course of drug effects. NOSTIM trials were not included in the calculation of inter-trial intervals. Intertrial intervals were variable and averaged 20 s. Total session duration was 30.5 min.

5. Data analysis: PPI was defined as 100-[(startle amplitude on prepulse trials / startle amplitude on P-ALONE trials) × 100], and was analyzed by mixed design ANOVAs. All data was inspected for the presence of "non-responders" defined by a mean startle response to P-ALONE trials of < 10 units. Other ANOVAs were used to assess P-ALONE magnitude, or NOSTIM trials. In all cases, analyses of NOSTIM trials revealed expected effects of DA agonists (Mansbach et al., 1988; Weber and Swerdlow, 2008), and no informative interactions with pretreatments (WC10 or WC44), and thus are not reported here in detail. Results from the two test compounds in the APO and PRA assays were directly compared within the same ANOVA in order to base the selection of the most promising compound for subsequent testing and optimization in medical chemistry on a clear scientific rationale. Post-hoc comparisons were conducted using

Fisher's PLSD. Data were collapsed across prepulse intensities and PPI blocks. Alpha was 0.05.

D. Results:

1. L741,626 vs. APO and PRA: In Weber et al. (2008a), a threshold dose of 1 mg/kg of the D2 receptor antagonist L741,626 significantly opposed PPI deficits induced by APO, but not those induced by PRA. This finding was confirmed in the present study (n=8 rats/dose of L741,626).

ANOVA of %PPI revealed significant main effects of APO dose (F=39.7, df 1,14, p<0.0001) and L741,626 dose (F=15.0, df 1,14, p=0.0017), and the critical APO × L741,626 dose interaction effect (F=5.3, df 1,14, p=0.037). Post-hoc tests revealed that APO significantly reduced %PPI in rats treated with 0 mg/kg of L741,626 (p=0.0013), and this effect was significantly opposed by 1 mg/kg L741,626 (p=0.0045; Fig. 5.2A). Yet, in rats treated with 1 mg/kg of L741,626, significant differences between vehicle and APO treated rats remained (p=0.0089), indicating that this dose of L741,626 did not *fully* prevent APO-induced PPI deficits. ANOVA of startle magnitude did not reveal any significant main or interaction effects (F<1 in all cases: inset, Fig. 5.2A), showing that drugs effects on startle magnitude cannot account for their significant effects on PPI.

In contrast, ANOVA of %PPI revealed a significant main effect of PRA dose (F=36.0, df 1,14, p<0.0001), but not of L741,626 dose (F<1), and no PRA × L741,626 dose interaction (F=1.6, df 1,14, ns; Fig. 5.2B). ANOVA of startle magnitude revealed a

significant main effect of PRA dose (F=26.7, df 1,14, p<0.0001). No main effect of L741,626 dose (F<1), and no PRA × L741,626 interaction (F=1.5, df 1,14, ns; inset, Fig. 5.2B) were detected. While the magnitude of the PRA-induced suppression of startle magnitude precluded the generation of subgroups matched for startle magnitude, simple regression analysis showed that PRA effects on startle magnitude contributed to less than 1% of the variance of PRA effects on PPI, suggesting that PRA effects on these two measures were independent (regression weights not significant). ANOVAs of %PPI were repeated, adding a factor for low vs. high levels of PRA-induced startle suppression, based on median split analyses. No critical main or interaction effects of %PPI differed between subgroups with low vs. high levels of PRA-induced startle suppression were detected.

Figure 5.2: Effects of a threshold dose of the preferential D2 antagonist L741,626 on PPI deficits induced by APO (A) and PRA (B). (A) ANOVA of %PPI revealed effects of APO (p<0.0001) and L741,626 (p<0.005), and the critical APO × L741,626 interaction (p<0.05). Post-hoc tests revealed that APO reduced %PPI in rats treated with 0 mg/kg of L741,626 (p<0.005), and this effect was opposed by 1 mg/kg L741,626 (p<0.005). ANOVA of startle magnitude did not reveal any main or interaction effects (inset). (B) ANOVA of %PPI revealed an effect of PRA (p<0.0001), but not of L741,626 dose (ns), and no PRA × L741,626 dose interaction effect (ns). ANOVA of startle magnitude revealed a significant main effect of PRA dose (p<0.0001). Drug effects on startle were dissociable from drug effects on PPI (see results). The star symbols denote a significant difference (p<0.005) between APO treated rats pre-treated with 0 vs. 1 mg/kg of L741,626.

2. WC44 and WC10 vs. APO and PRA: *In vitro* assays have characterized WC44 as a full D3 receptor agonist (Chu et al., 2005). We therefore tested the effects of WC44 alone on %PPI (n = 4/WC44 dose). ANOVA of %PPI revealed no significant main effect WC44 dose (F<1; Fig. 5.3) indicating that WC44 does not have a DA agonist-like effect in this *in vivo* assay. ANOVA of startle magnitude revealed a significant main effect of WC44 dose (F=4.5, df 3,12, p=0.025; Fig. 5.3 inset), with higher startle magnitudes for 1 mg/kg of WC44 when compared to vehicle (p=0.030), but not for any of the other active WC44 doses. Thus, at doses that are bioactive, WC44 does not exhibit DA agonist-like effects on PPI.

Figure 5.3: Effects of the presumed D3 agonist WC44 on PPI and startle magnitude (insets). ANOVA of %PPI in SD rats revealed no significant main effect WC44 dose indicating that WC44 does not have an agonist-like effect *in vivo*. ANOVA of startle magnitude revealed a significant main effect of WC44 dose.
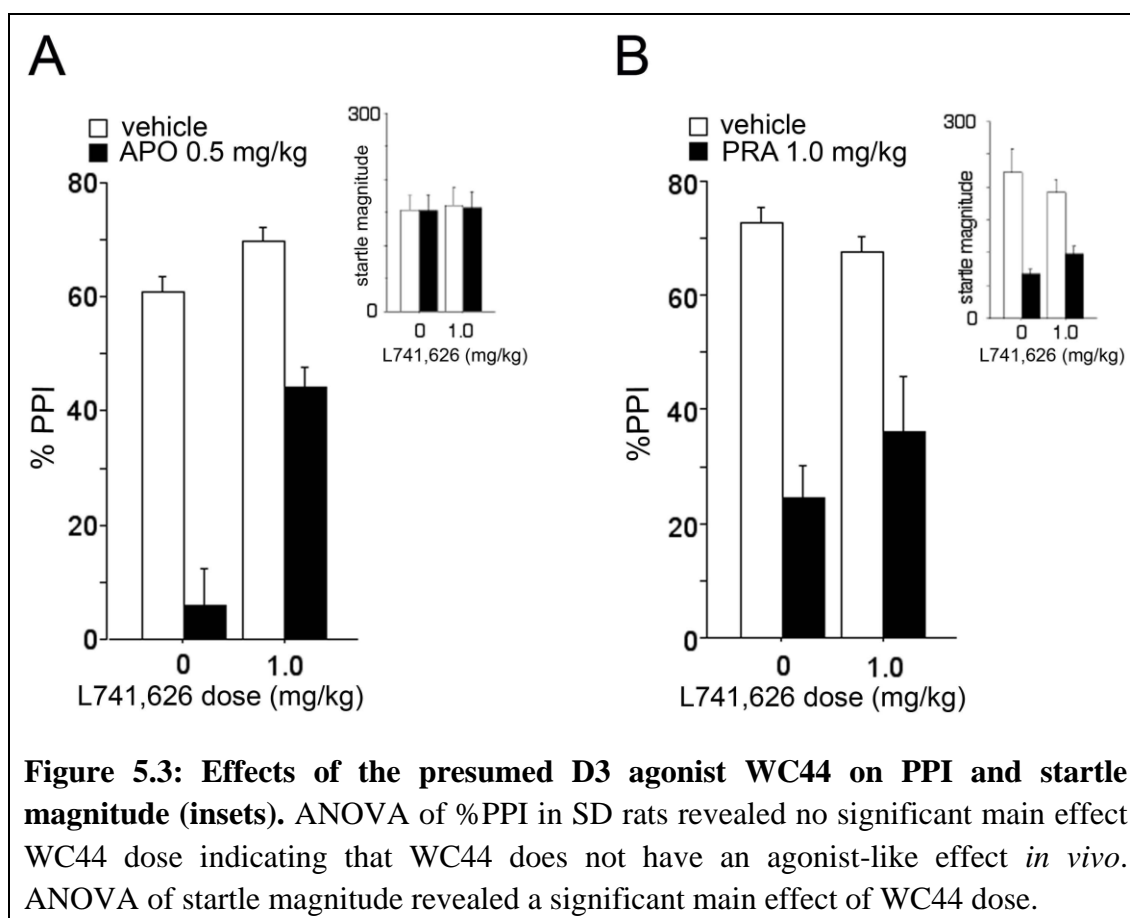
We then directly compared the activity of WC10 (n = 8/ WC10 dose) and WC44 (n = 6/ WC44 dose) in the APO assay. ANOVA revealed a significant main effect of APO dose (0 vs. 0.5 mg/kg, F=183.9, df 1,24, p<0.0001), but no main effect of pretreatment type (WC44 vs. WC10; F=2.3, df 1,24, ns), and no effect of pretreatment dose (0 vs. 10 mg/kg of either test compound; F=1.6, df 1,24, ns). There were no significant interactions of pretreatment type × pretreatment dose (F=3.3, df 1,24, p=0.081), or pretreatment type × APO (F<1, ns). There was a significant interaction of APO × pretreatment dose (F=4.6, df 1,24, p=0.042), and most importantly APO × pretreatment type × pretreatment dose (F=6.9, df 1,24, p=0.015), indicating that WC10 and WC44 differed in their impact on APO-induced PPI deficits. To understand the basis for this interaction, separate ANOVAs were conducted for WC44 and WC10. ANOVA of %PPI for WC44 revealed a significant main effect of APO dose (F=72.4, df 1,14, p<0.0001), but no effect of WC44 dose and no APO × WC44 dose (F<1 for all cases) indicating that WC44 does <u>not</u> reverse APO-induced PPI deficits. Post-hoc tests revealed that APO significantly reduced %PPI in rats pretreated with 0 mg/kg of WC44 (p=0.0011) and in rats pretreated with 10 mg/kg WC44 (p=0.0026), and that among APO-treated rats, %PPI did not differ between those pretreated with 0 vs. 10 mg/kg WC44 (ns). ANOVA of %PPI for WC10 revealed a significant main effect of APO dose (F=115.2, df 1,14, p<0.0001) and WC10 dose (F=5.8, df 1,14, p=0.031), and most importantly a significant APO × WC10 interaction (F=16.5, df 1,14, p=0.0012). Post-hoc tests revealed that APO significantly reduced %PPI in rats treated with 0 mg/kg of WC10 (p<0.0001), and this effect was significantly opposed by 10 mg/kg WC10 (p=0.0051),

indicating that WC10 opposed APO-induced PPI deficits (Fig. 5.4A). Yet, in rats treated with 10 mg/kg of WC10 a significant difference between rats treated with vehicle and APO remained (p=0.0042), indicating that this dose of WC10 did not *fully* prevent PRA-induced PPI deficits.

ANOVA of startle magnitude revealed a significant main effect of pretreatment type (WC44 vs. WC10, F=8.7, df 1,24, p=0.007), reflecting reduced startle amplitudes in the WC10 experiment. All other effects were not statistically significant (Fig 5.4A inset). Importantly, the effect of pretreatment type on startle magnitude is neither due to APO, WC10, WC44, nor a combination thereof and therefore cannot account for the critical interaction of APO × pretreatment type × pretreatment dose in PPI measures described above. Concurrently, in subsets of animals from both experiments that were matched for mean startle magnitude (effect of experiment: F<1), the critical interaction of APO × pretreatment type × pretreatment dose in measures of PPI was still apparent (p=0.014), with identical post-hoc patterns to those detected in the inclusive sample.

Figure 5.4: Effects of WC44 (A) and WC10 (B) on PPI deficits induced by APO.
ANOVA of %PPI revealed an APO dose effect (p<0.0001), an APO × pretreatment-dose effect (p<0.05), and an APO dose × pretreatment dose × pretreatment type effect (p<0.05). Separate ANOVAs for the WC44 and WC10 experiment revealed a main effect of APO in both experiments (p<0.0001, each). In addition, an APO dose × pretreatment dose effect for WC10 (p<0.005), but not for WC44 (n.s.), indicative of reversal of APO-induced PPI deficits in rats treated with WC10, but not WC44. ANOVA of startle magnitude revealed a main effect of pretreatment type (p<0.01) indicating lower startle magnitudes in the WC10 experiment; all drug-related main or interaction effect were non-significant, indicating that drug effects on PPI were dissociable from drug effects on startle magnitude. The star symbol denotes significant differences (p<0.05) between APO rats pre-treated with 0 vs. 10 mg/kg of WC10.

Parallel analyses were completed (n=12 /WC10 dose and n=12/ WC44 dose) using PRA to disrupt PPI instead of APO. ANOVA revealed a significant main effect of PRA dose (0 vs. 1 mg/kg, F=46.9, df 1,44, p<0.0001), and pretreatment dose (0 vs. 10

mg/kg of either test compound; F=9.5, df 1,44, p=0.0035), but no main effect of pretreatment type (WC44 vs. WC10; F<1). Importantly, there was a significant effect of PRA × pretreatment dose (F=6.5, df 1,44, p=0.015), but no interaction of PRA × pretreatment type (F=1.7, df 1,44, ns), no pretreatment type × pretreatment dose interaction (F<1), and no PRA × pretreatment type × pretreatment dose interaction (F<1), indicating that the two test compounds did not differ significantly from each other in their ability to reverse these PRA-induced PPI deficits. A post-hoc comparison in PRA-treated rats across compounds revealed that, compared to the 0 mg/kg pretreatment dose, the 10 mg/kg dose significantly increased PPI (F=9.9, df 1,46, p=0.0029). Separate ANOVAs were then conducted for WC44 and WC10. ANOVA of %PPI for WC44 revealed a significant main effect of PRA dose (F=26.6, df 1,22, p<0.0001), a trend towards a WC44 dose effect (F=3.5, df 1,22, p=0.073), and a significant PRA × WC44 dose interaction (F=4.5, df 1,22, p=0.047), reflecting the fact that WC44 does reverse PRA-induced PPI deficits. Post-hoc tests revealed that PRA significantly reduced %PPI in rats treated with 0 mg/kg of WC44 (p=0.0011), and this effect was opposed by 10 mg/kg WC44 (p=0.045). Yet, in rats treated with 10 mg/kg of WC44, significant differences between vehicle and PRA treated rats remained (p=0.018), indicating that this dose of WC44 did not *fully* prevent PRA-induced PPI deficits. ANOVA of %PPI for WC10 revealed a significant main effect of PRA dose (F=20.4, df 1,22, p=0.0002) and WC10 dose (F=6.4, df 1,22, p=0.019), while the PRA × WC10 interaction effect did not reach significance (F=2.0, df 1,22, ns). Post-hoc tests, however, revealed that PRA significantly reduced %PPI in rats treated with 0 mg/kg of WC10 (p=0.0004), and this effect was significantly opposed by 10 mg/kg WC10 (p=0.032; Fig. 5.5A). Similarly to the results

obtained with WC44, however, in rats treated with 10 mg/kg of WC10, there was a trend towards differences between vehicle and PRA treated rats (p=0.082), suggesting that this dose of WC10 did not *fully* prevent PRA-induced PPI deficits.

The corresponding ANOVA of startle magnitude based on the comparison of 0 vs. 10 mg/kg of the test compounds revealed a significant main effect of PRA dose (F=56.1, df 1,44, p<0.0001). All other main or interaction effects were not statistically significant (Fig. 5.5B inset). While the magnitude of the startle suppression induced by PRA did not allow to create subgroups of rats matched for startle magnitude, simple regression analyses based on the comparison of 0 vs. 10 mg/kg of the test compounds showed that the PRA effect on startle amplitude accounted for less than 2 percent of the corresponding PPI effects, suggesting that these two measures were independent (regression weights ns). To further assess this issue, all ANOVAs of %PPI were repeated, adding a factor for low vs. high levels of PRA-induced startle suppression, based on median split analyses. No critical main or interaction effects of %PPI differed between subgroups with low vs. high levels of PRA-induced startle suppression.

Figure 5.5: Effects of WC44 (A) and WC10 (B) on PPI deficits induced by PRA, and startle magnitude (insets). ANOVA of %PPI revealed effects of pretreatment dose (p<0.05), PRA dose (p<0.0001), PRA × pretreatment type effect (p<0.05) and the crucial PRA × pretreatment dose effect (p<0.05), but no PRA × pretreatment type × pretreatment dose effect (n.s.). ANOVA of startle magnitude revealed a PRA dose effect, but no main effect or interaction effect with pretreatment dose, or pretreatment type, indicating that drug effects on startle magnitude cannot account for the prevention of PRA-induced PPI deficits by either test compound. Star symbols denote significant differences (p<0.05) between PRA treated rats pre-treated with 0 vs. 10 mg/kg of either WC10 or WC44.

E. Discussion:

In the present study, measures of PPI deficits were used to identify novel test compounds that act as preferential D3-receptor linked antagonists in rats. WC10 and WC44, two representatives of a novel panel of putative D3 selective compounds, were

characterized in this assay. A D1/D2/(D3) related mechanism of action was identified for WC10. A novel preclinical profile based on a functional D3-receptor antagonist mechanism of action was identified for WC44.

Many groups have demonstrated that APO-induced PPI deficits are opposed by typical and atypical antipsychotics (cf. Swerdlow et al., 2008b). We have also reported PPI-disruptive effects of the preferential D3 receptor agonist PRA in rats (Weber et al., 2008a). This effect was confirmed in the present study, consistent with studies using other preferential D3 agonists (Caine et al., 1995; Swerdlow et al., 1998a; Varty and Higgins, 1998; Zhang et al., 2007). Our previous studies have further demonstrated a greater sensitivity of the preferential D2 receptor antagonist L741,626 to reverse APO-induced PPI deficits vs. PRA-induced PPI deficits, suggesting that, unlike APO, PRA effects on PPI are not mediated by D2 receptors (Weber et al., 2008a). These findings were also confirmed here (Fig. 5.2). The present study used these apparent differences in D3 vs. D2 involvement in PRA- vs. APO-induced PPI deficits to characterize WC10 and WC44, two novel D3-receptor selective compounds, and to predict novel preclinical antipsychotic profiles consistent with a predominant D3 receptor-linked mechanism of action. Zhang et al. (2007) used a similar strategy to compare the effects of preferential D3 antagonists in assays of PPI deficits induced by either APO, or the preferential D3 agonist PD128,907 in rats. As large clinical trials for D3 preferential antagonists have not been published to date, we cannot conclude that the high antipsychotic predictive validity of the APO/PPI for mixed D2/D3 antagonists extends to the PRA/PPI assay for the detection of potentially novel antipsychotics with a preferential D3-receptor linked

mechanism of action. However, at the very least, our findings and those of Zhang et al., (2007) show that this use of the PPI assays can detect apparent differences in functional D3 antagonism *in vivo*.

WC10 and WC44 had very distinct profiles in the APO-PPI assay: WC10 significantly opposed APO-induced PPI deficits, while WC44 did not. This profile of WC10 is shared with both typical and atypical antipsychotics (Swerdlow et al., 1994; cf. Geyer et al., 2001; Swerdlow et al., 2008b), while the inactivity of WC44 in this assay parallels that reported with the preferential D3 antagonists A-691990 and SB-277011 (Zhang et al., 2007). The prevention of APO-induced PPI deficits *per se* does not rule out a D3-receptor related mechanism of action, as the PPI-disruption caused by a APO is likely to involve both D3 and D2, as well as other DA receptor subtypes (for the receptor binding profile of APO see e.g.: Millan et al., 2002). Consistent with this, Millan et al. (2008b) reported that the highly preferential D3 antagonist S33138 significantly opposed APO-induced PPI deficits in rats. To our knowledge, no study has yet compared the effects of S33138 on PPI deficits induced by APO- vs. those induced by a preferential D3 agonist, like PRA, PD128,907, or 7-OHDPAT.

In the PRA/PPI assay, both WC10 and WC44 opposed PRA-induced PPI deficits. Previous studies have shown that PPI deficits induced by preferential D3 agonists such as PD128,907 and 7-OHDPAT can be prevented by preferential D3 receptor antagonists such as A-691990 and SB-277011 (Zhang et al., 2007) as well as by the mixed D2-like antagonist such as HAL (Caine et al., 1995; Zhang et al., 2007). This shows that the prevention of PRA-induced PPI-deficits per se does not indicate a novel antipsychotic-

like profile. *However, the combination of inactivity in the APO/PPI assay, and activity in the PRA/PPI assay, suggest that WC44 has properties of a novel, D3 preferential antagonist.* Arguably, no full reversal of the PRA-induced PPI deficits was achieved with either test compound in the dose range tested, suggesting that higher doses should be tested (see below), or that more efficacious compounds are needed. We are currently studying variants of WC44 with the goal to achieve increased efficacy in this model.

In the *in vitro* assay used by Chu et al. (2005), the effects of WC44 were tested in the absence of DA, using forskolin-stimulated adenylate cyclase activity; WC44 yielded 92% of the response induced by quinpirole and was hence classified as a full D3 agonist. The contrast of the agonist-like profile for WC44 in these *in vitro* studies vs. the lack of agonist-like effects in the *in vivo* findings reported here (Fig. 5.3) is intriguing. We cannot exclude the possibility that WC44 could have DA agonist-like effects at a higher dose range, but the range used here includes the IC_{50} of 5.5 mg/kg for the suppression of abnormal involuntary movements (AIM) in the same rat strain (Kumar et al., 2009). In ongoing studies, we have detected no independent effects of 20 mg/kg WC44 on PPI, but a near-complete opposition of PRA-induced PPI deficits (in preparation). While we have no immediate explanation for the lack of agonist-like *in vivo* effects of WC44 at present, one explanation may be the presence of endogenous DA *in vivo*. Among all DA receptors, D3 receptors have the highest DA affinity with a K_i in the range of extrasynaptic and intrasynaptic DA levels (cf. Richtand, 2006). This suggests that D3 receptors are likely to be (partially) occupied by DA *in vivo*. Hence, agonist-like effects derived under *in vitro* conditions in the absence of DA may not translate to *in vivo*

findings for compounds with (even weak) partial agonist-like properties relative to DA. A second explanation may lie in the concept of functional selectivity, i.e. the phenomenon that a compound acting via a single receptor type can have diverse functional properties ranging from full agonist, to partial agonist, to full antagonist, depending on the intracellular signaling cascade that is activated downstream of the receptor (Mailman, 2007).

While both WC10 and WC44 have been thoroughly characterized in *in vitro* binding studies for DA, serotonin, and sigma receptors, and in functional assays for D2/D3 receptors (Chu et al., 2005), it is not known at present whether these compounds have functional activity on non-DA receptors. For example, a relatively high binding affinity was detected for 5-HT1a receptors for both compounds, but the functional consequences of these binding properties are not yet known. 5-HT1a agonists disrupt PPI in rats (Rigdon and Weatherspoon, 1992), but neither WC10, nor WC44 disrupted PPI in the present study. Thus, the most parsimonious explanation of the present data is that the effects detected with WC10 and WC44 reflect DA-linked mechanisms of these test compounds.

In summary, studies detected D2 and D3 antagonist profiles for WC10, and findings suggested that WC44 may functionally oppose D3 but not D2 receptor activation. Based on this latter profile, WC44 might have a novel antipsychotic profile linked to functional D3 receptor antagonism. These initial studies cannot rule out a potential role of other receptors types for which the binding affinities of WC10 and WC44 are not yet known. Other test compounds with putative functional D3 antagonism

are being evaluated in this assay, with the goal of predicting their potential for clinical applications.

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CHAPTER 6:

Heritable strain differences in sensitivity to the startle gating-disruptive effects of D2 but not D3 receptor stimulation

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A. Abstract:

Prepulse inhibition of the startle reflex (PPI) is an operational measure of sensorimotor gating that is deficient in several brain disorders and is disrupted in rats by dopamine agonists. There are robust heritable strain differences between Sprague Dawley (SD) and Long Evans (LE) strains in the sensitivity to the PPI-disruptive effects of dopamine agonists associated with differential gene expression in the nucleus accumbens. Here we compared the contribution of D2 vs. D3 receptors to this heritable difference, using the D3-preferential agonist, pramipexole, the mixed D3/D2–agonist, quinpirole, the mixed D1/D2-like agonist, apomorphine, and the preferential D2 antagonist L741,626. All DA agonists disrupted PPI in SD and LE rats. Greater SD than LE sensitivity for this effect was evident with apomorphine and quinpirole, but not pramipexole. The selective D2 antagonist L741,626 preferentially reversed apomorphine-induced PPI deficits at a dose that did not alter pramipexole-induced PPI deficits. We conclude that the heritable pattern of greater PPI "disruptability" by DA agonists in SD vs. LE rats reflects differences in D2 but not D3 receptor-associated mechanisms.

B. Introduction:

Evidence suggests that vulnerability for developing schizophrenia can be inherited (Harrison and Weinberger, 2005; Sullivan, 2005) and that genes conferring this vulnerability ultimately do so via changes in brain circuitry. Great effort is being put towards identifying the genetic basis of this vulnerability through the use of endophenotypes, i.e. phenotypes that are intermediate between the genes and the more complex clinical manifestations of these diseases (Gottesman and Gould, 2003; Turetsky et al., 2007). One useful schizophrenia endophenotype may be reduced PPI of the startle reflex (Graham, 1975). Normal prepulse inhibition of startle (PPI) is a cross-species phenomenon that also occurs in humans, rats, and mice when a weak lead stimulus inhibits the response to an intense, abrupt startling stimulus. PPI is reduced in schizophrenia patients and their unaffected first-degree relatives (Braff et al., 1978, 2001; Cadenhead et al., 2000; Kumari et al., 2005) suggesting that deficient PPI may be a useful endophenotype for inherited forms of schizophrenia.

In rats, PPI is potently disrupted by dopamine (DA) receptor agonists, including the mixed D1/D2-like agonist, apomorphine (APO) and the mixed D3/D2–agonist, quinpirole (QUIN; Mansbach et al., 1988, Peng et al., 1990; Swerdlow et al., 1986; cf. Geyer et al., 2001). Sensitivity to these PPI-disruptive effects of some DA agonists differs across rat strains. For example, Sprague Dawley rats from Harlan Laboratories (SD) are significantly more sensitive to the PPI-disruptive effects of APO, QUIN, and the indirect DA agonist amphetamine (AMPH), compared to Long Evans rats from Harlan Laboratories (LE; Swerdlow et al., 2001c, 2003b, 2004a, 2004b, 2004c; Weber and

Swerdlow, 2008). These differences are innate (Swerdlow et al., 2004a, 2004c), neurochemically specific (Swerdlow et al., 2003b, 2004b), independent of stimulus modality (Weber and Swerdlow, 2008) and cannot be explained by differences in maternal behavior (Swerdlow et al., 2004a). Finally, these strain differences appear to be linked to inherited properties of DA-linked G-protein function (Swerdlow et al., 2006a), and to differential nucleus accumbens gene expression, particularly among genes associated with DA signaling pathways (Shilling et al., 2008). Conceivably, this heritable strain difference in the "disruptability" of PPI by DA activation may provide a useful model for understanding the basis for reduced PPI in heritable, DA-linked brain disorders such as schizophrenia (Braff et al., 1978) and Tourette Syndrome (Castellanos et al., 1996).

While existing data suggest a role of D2-family receptors in this model, we do not know which D2 receptor subtype (D2, D3 or D4) is responsible for the inherited phenotype of PPI "disruptability". Hence, in the present study, we tested whether SD vs. LE rats differed in sensitivity to the PPI-disruptive effects of the preferential D3 agonist pramipexole, in addition to the mixed D3/D2–agonist QUIN, and the mixed D1/D2-like agonist APO. To parse the potential contribution of D2 receptors to the PPI-disruptive effects of APO and pramipexole, these effects were also tested after pretreatment with the preferential D2 receptor antagonist L741,626 (Millan et al., 2000c) in SD rats.

C. Methods:

1. Experimental animals: Adult male SD (n = 102) and LE (n = 38) rats (225-250 g; Harlan Laboratories, Livermore, CA) were housed in groups of 2-3 animals per cage, and maintained on a reversed light/dark schedule with water and food available *ad libitum*. Rats were handled within 2 d of arrival. Testing occurred during the dark phase. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and were approved by the UCSD Animal Subjects Committee (protocol #S01221).

2. Drugs: Apomorphine hydrochloride hemihydrate, quinpirole hydrochloride, 85% (w/v) lactic acid solution, and 1N NaOH solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pramipexole hydrochloride was purchased from Toronto Research Chemicals (North York, On, Canada), and L741,626 was purchased from Tocris (Ellisville, MO, USA). Drug doses are based on mg/kg of salts. All drugs were administered subcutaneously (sc) in a volume of 1 ml/kg. Based on pilot studies, PRA (saline, 0.03, 0.06, 0.1 or 1.0 mg/kg) was administered 30 min prior to PPI testing, QUIN (saline vehicle or 0.5 mg/kg) was administered 10 min prior to PPI testing, and APO (0.01 % ascorbate/saline vehicle, 0.1 or 0.5 mg/kg) was administered immediately prior to PPI testing. L741,626 (water vehicle, 1, 3, or 10 mg/kg) was dissolved in 0.05-0.5% lactic acid/water (w/v) and pH was adjusted to ≥ 5 using NaOH. L741,626 was administered 30 min prior to PRA or APO treatment.

3. Apparatus: Startle chambers for rats (San Diego Instruments, San Diego, CA, USA) were housed in a sound-attenuated room, and consisted of a Plexiglas cylinder 8.2 cm in diameter resting on a 12.5×25.5 cm Plexiglas frame within a ventilated enclosure. Noise bursts were presented via a speaker mounted 24 cm above the cylinder. A piezoelectric accelerometer mounted below the Plexiglas frame detected and transduced motion from within the cylinder. Stimulus delivery was controlled by the SR-LAB microcomputer and interface assembly, which also digitized (0-4095), rectified, and recorded stabilimeter readings. One hundred 1-ms readings were collected beginning at stimulus onset. Startle amplitude was defined as the average of the 100 readings.

4. Startle testing procedure: Approximately 7 days after shipment arrival, rats were exposed to a short "matching" startle session. They were placed in the startle chambers for a 5 min acclimation period with a 70 dB(A) background noise, and then exposed to a total of 17 P-ALONE trails (40 ms - 120 dB(A) noise bursts) that were interspersed with 3 PP12dB+P-ALONE trials (P-ALONE preceded 100 ms (onset-to-onset) by a 20 ms noise burst of 12dB above background). Rats were assigned to drug dose groups based on average %PPI from the matching session to ensure similar baseline PPI levels between groups. The number of groups created in the matching session equaled the number of dose groups per strain. Average PPI in these groups was 55 % for SD rats and 57 % for LE rats. For each experiment and strain, average %PPI of the dose groups typically differed by less than 3%. Group sizes ranged from 2 to 4 rats/strain/dose group/experimental day. All DA agonist studies (APO, PRA, QUIN) used within-

subjects, balanced dose order designs. The number of injections administered to each rat -

and the number of experimental days needed to complete the experiment - equaled the

number of doses of the DA agonist (including vehicle treatment) tested. The sequence of

drug doses administered was counterbalanced between animals to control for any effects

of treatment day or sequence. Tests of L741,626 against APO or PRA were two day-

studies and had a mixed-model, balanced dose-order design with L741,626 as the

between factor and APO or PRA as the within factor. Inter-test intervals were 3-5 days

for PRA and APO agonist studies, and 6-7 days when PRA and APO were tested against

L741,626. Inter-test interval was 11 days for QUIN, to minimize the known long-term

effects of QUIN on D2 function and PPI (Culm and Hammer, 2004).

Starting 4 d after the matching session, the effects of PRA were studied in two

separate groups of rats. One group of rats received a low dose range (0, 0.03 and 0.06

mg/kg), and another group of rats received a higher dose range (0, 0.1 and 1.0 mg/kg).

Each of these PRA experiments was followed by an APO experiment, beginning 4 days

after the final injection of the PRA experiment using the same rats. For the APO

experiment, rats were treated on two days with either vehicle or 0.5 mg/kg APO. A

separate group of rats was tested on two days, with either vehicle or 0.5 mg/kg QUIN.

Three experiments with L741,626 were conducted. First, PPI was tested after

pretreatment with L741,626 (0, 1, 3, 10 mg/kg) and treatment with APO (0 vs. 0.5

mg/kg). Second, in separate rats, PPI was tested after pretreatment with L741,626 (0 or 1

mg/kg) and treatment with a low dose of APO (0 vs. 0.1 mg/kg). Third, in separate rats,

PPI was tested after pretreatment with L741,626 (0, 1, 3 or 10 mg/kg) and treatment with

PRA (0 vs. 1.0 mg/kg). Pretreatment time for L741,626 relative to APO or PRA treatment was 30 min.

Immediately (APO), 10 min (QUIN), or 30 min (PRA) after injection of the DA agonists, rats were placed in the startle chambers for a 5 min acclimation period with a 70 dB(A) background noise. They were then exposed to a series of trial types which were presented in pseudorandom order: (1) P-ALONE ALONE (a 40 ms, 120 dB(A) noise burst); (2-4) P-ALONE preceded 100 ms (onset-to-onset) by a 20 ms noise burst of either 5 (PP5dB+P-ALONE), 10 (PP10dB+P-ALONE), or 15 dB above background (PP15dB+P-ALONE). Interspersed between these trials were trials in which no stimulus was presented, but cage displacement was measured (NOSTIM trials). The session began with 4 consecutive P-ALONE trials and ended with 3 consecutive P-ALONE trials; between these trials were two blocks, each consisting of 8 P-ALONE trials, 5 PP5dB+P-ALONE trials, 5 PP10dB+P-ALONE trials, and 5 PP15dB+P-ALONE trials. Intertrial intervals were variable and averaged 15 s. NOSTIM trials were not included in the calculation of inter-trial intervals. Total session duration was 18.25 min.

5. Data analysis: PPI was defined as 100-[(startle amplitude on prepulse trials / startle amplitude on P-ALONE trials) $\times$ 100], and was analyzed by mixed design ANOVAs. Other ANOVAs were used to assess P-ALONE magnitude, or NOSTIM trials. Post-hoc comparisons were conducted using Fisher's PLSD. The magnitude of drug effects on PPI was calculated as "%PPI$_{vehicle}$-%PPI$_{drug}$". ANCOVAs were used to assess

the effect of baseline PPI differences on QUIN-induced PPI effects with strain as a covariate. Regressions were based on simple linear models. Where group differences were detected in P-ALONE magnitude or baseline (vehicle) PPI, several approaches were used to parse these effects from those in PPI-drug sensitivity. Data were collapsed across prepulse intensities. Alpha was 0.05.

D. Results:

1. APO effects: ANOVA of %PPI revealed significant main effects of strain (F=6.16, df 1,42, p<0.02) and APO dose (F=198.35, df 1,42, p<0.0001), and a significant strain × dose interaction (F=39.72, df 1,42, p<0.0001). Separate ANOVAs for each strain revealed significant effects of APO dose in SD rats (F=139.05, df 1,21, p< 0.0001) and LE rats (F=59.88, df 1,21, p< 0.0001). Importantly, the magnitude of the APO effect (%$PPI_{vehicle}$-%PPI_{APO}) was significantly larger in SD than LE rats (p<0.0001), indicating greater sensitivity to the PPI-disruptive effects of APO in SD than LE rats (Fig. 6.1A).

Based on the significant main effect of strain on PPI, a separate analysis was conducted for subsets of SD and LE rats that were matched for baseline PPI levels. Subsets were constructed by eliminating the extreme responders until baseline PPI was numerically balanced between strains (mean (SEM) of vehicle PPI: SD: 73.0 (1.4), n=12; LE: 72.2 (1.6), n=12). ANOVA of %PPI in these subgroups confirmed the key results: a significant strain × dose interaction (F=14.31, df 1,22, p<0.002), with a significantly greater APO effect (%$PPI_{vehicle}$-%PPI_{APO}) in SD than LE rats (p<0.002).

ANOVA of startle magnitude on P-ALONE trials revealed significant main effects of strain (F=6.82, df 1,42, p<0.02) and APO dose (F=8.19, df 1,42, p<0.01), and a significant strain × dose interaction (F=14.80 df 1,42, p<0.001). This interaction reflected significant startle-enhancing effects of APO in LE rats (F=17.05, df 1,21, p<0.001), but not in SD rats (F<1). Baseline (vehicle) levels of startle magnitude were almost identical across strains (mean (SEM): SD rats = 170.4 (18.9); LE rats = 176.6 (24.4)) (Fig. 6.1A, inset).

To assess the impact of the startle-enhancing effects of APO on PPI strain differences, subgroups were matched as above, such that the effects of APO on startle magnitude were numerically balanced between strains (mean (SEM) startle magnitude: SD (n=20) vehicle vs. APO = 161.8 (19.8) vs. 160.7 (30.3); LE (n=8) vehicle vs. APO = 237.0 (50.9) vs. 242.5 (54.1)). ANOVA of P-ALONE amplitude revealed no significant main effect of strain (F=2.70, df 1,26, ns) or APO dose (F<1), and no strain × dose effect (F<1). ANOVA of %PPI in these subgroups again confirmed the key results: a significant strain × dose interaction (F=14.53, df 1,26, p<0.001), with a significantly greater APO effect (%PPI$_{vehicle}$-%PPI$_{APO}$) in SD than LE rats (p<0.001).

Inspection of NOSTIM levels revealed very small signal amplitudes (< 1% of pulse alone amplitudes), yet a significant APO × strain interaction (F=10.85, df 1,42, p<0.01), with greater APO responsiveness in SD than LE rats. Simple regression showed that this effect contributed less than 2% of the variance to the observed APO effects on PPI in SD rats, and less than 11% in LE rats (regression weights ns).

2. QUIN effects: ANOVA of %PPI revealed no significant main effect of strain (F<1), a significant main effect of QUIN dose (F=54.41, df 1,30, p<0.0001), and a significant strain × dose interaction (F=6.87, df 1,30, p<0.02). Separate ANOVAs for each strain revealed significant effects of QUIN dose in SD rats (F=33.03, df 1,15, p< 0.0001) and LE rats (F=23.24, df 1,15, p< 0.0005). Importantly, the magnitude of the QUIN effect (%PPI$_{vehicle}$-%PPI$_{QUIN}$) was significantly larger in SD than LE rats (p<0.02), indicating greater sensitivity to the PPI-disruptive effects of QUIN in SD than LE rats (Fig. 6.1B). While the ANOVA detected no main effect of strain on PPI (F<1), an analysis of covariance of the QUIN PPI effect (%PPI$_{vehicle}$-%PPI$_{QUIN}$) was conducted, with baseline (vehicle) PPI as a covariate and strain as a between factor. This confirmed a significant effect of strain (F=4.87, df 1,28, p<0.05). Thus, SD rats are more sensitive than LE rats to the PPI-disruptive effects of QUIN, even when controlling for any variance linked to levels of baseline PPI.

Because Culm and Hammer (2004) demonstrated lasting effects of QUIN treatment on D2 function and PPI, we examined QUIN effects limited to the initial drug test day. ANOVA confirmed the critical findings: no significant effect of strain (F<1), but a significant strain × QUIN interaction (F=5.38, df 1,28, p<0.03). Because data from this single test day did not permit calculation of a "QUIN PPI effect", we compared PPI in QUIN-treated SD vs. LE rats; this revealed a significant main effect of strain (p<0.05), confirming that LE rats exhibited higher PPI levels after QUIN (i.e. were less inhibited by QUIN) compared to SD rats.

ANOVA of startle magnitude on P-ALONE trials revealed no significant main effect of strain (F=2.06, df 1,30, ns) or QUIN dose (F=3.06, df 1,30, 0.05<p<0.1), or strain × dose interaction (F=3.18 df 1,30, 0.05<p<0.1). This trend towards an interaction reflected startle-reducing effects of QUIN in SD rats (F=11.34, df 1,15, p<0.005), but not in LE rats (F<1). Baseline (vehicle) levels of startle magnitude were almost identical across strains (mean (SEM): SD rats = 180.3 (18.9); LE rats = 178.2 (36.4)) (Fig. 6.1B, inset).

To assess the impact of QUIN effects on startle magnitude on PPI strain differences, subgroups were matched as above (mean (SEM) startle magnitude: SD (n=7) vehicle vs. QUIN = 119.4 (15.1) vs. 121.2 (24.4); LE (n=16) vehicle vs. QUIN = 178.2 (36.4) vs. 179.0 (27.0)). ANOVA of P-ALONE amplitude revealed no significant effect of strain (F=2.09, df 1,21, ns) or QUIN dose (F<1), and no strain × dose effect (F<1). ANOVA of %PPI in these subgroups confirmed a significant strain × dose interaction (F=6.34, df 1,21, p<0.05), with a significantly greater QUIN effect (%PPI$_{vehicle}$-%PPI$_{QUIN}$) in SD than LE rats (p<0.05).

Inspection of NOSTIM levels revealed very small signal amplitudes approximating or less than 1% of pulse alone amplitudes, yet a significant main effect of dose (F=48.76 df 1,30, p<0.0001), but no significant effect of strain (F<1), or strain × dose interaction (F<1). Simple regression showed that this effect contributed less than 7% of the variance to the observed QUIN effects on PPI in SD rats (regression weight ns), and less than 13% in LE rats (regression weight ns).

Figure 6.1: Effects of APO (A) or QUIN (B) on PPI and startle magnitude (insets) in SD and LE rats. Compared to LE rats, SD rats were significantly more sensitive to the PPI-disruptive effect of APO and QUIN. These effects persisted when basal (vehicle) PPI levels and APO or QUIN effects on startle magnitude were controlled by balancing subgroups or via an analysis of covariance (see text). ** $p<0.005$, *** $p<0.0001$.

3. PRA effects: ANOVA of %PPI for low dose ranges (0.03-0.06 mg/kg) revealed significant main effects of strain (F=53.59, df 1,22, $p<0.0001$) and PRA dose (F=11.33, df 2,22, $p<0.0002$), but no significant strain $\times$ dose interaction (F<1). The magnitude of the PRA effect (%PPI$_{vehicle}$-%PPI$_{PRA}$) in SD and LE rats was not significantly different for either 0.03 mg/kg (F=1.52, df 1,22, ns), or 0.06 mg/kg (F<1). Post-hoc analysis in SD rats revealed a trend towards reduced %PPI for the lowest dose of PRA (0.03 mg/kg; $0.05<p<0.1$) and significantly reduced %PPI for 0.06 mg/kg

(p<0.002) relative to vehicle. Post-hoc tests in LE rats revealed that %PPI was significantly reduced for 0.03 mg/kg and 0.06 mg/kg doses (both p<0.001) relative to vehicle (Fig. 6.2A).

Based on the significant main effect of strain on %PPI, a separate analysis was conducted for subsets balanced for levels of baseline PPI (mean (SEM) of vehicle PPI: SD: 70.7 (3.1), n=6; LE: 69.1 (4.6), n=6). These subgroups were also similar with respect to baseline (vehicle) startle magnitude (mean (SEM): SD rats: 137.2 (49.3); LE rats: 133.4 (27.5)). ANOVA of %PPI revealed significant main effects of strain (F=15.46, df 1,10, p<0.005) and PRA dose (F=7.25, df 2,20, p<0.005), and a significant strain × dose interaction (F=5.41, df 2,20, p<0.02). While the overall magnitude of the PRA effect on PPI (%PPI$_{vehicle}$-%PPI$_{PRA}$) was not significantly different across strains, sensitivity to the 0.03 dose was greater in LE than SD rats (F=13.39, d 1,10, p<0.005), and a similar trend was detected at 0.06 mg/kg (F=4.34; df 1,10, 0.05<p<0.1).

ANOVA of startle magnitude on P-ALONE trials in rats tested with low doses of PRA revealed a significant main effect of strain (F=7.36, df 1,22, p<0.02), but not dose (F=1.40, df 2,44, ns), and a significant dose × strain interaction (F=4.34, df 2,22, p<0.02). This interaction reflected a trend towards startle-enhancing effects of PRA in LE rats (F=3.10, df 2,22, 0.05<p<0.1), but not in SD rats (F=1.73, df 2,22 ns; Fig. 6.2A, inset). Post-hoc tests in LE rats revealed that startle magnitude was significantly enhanced by 0.03 mg/kg (p<0.05), but not by 0.06 mg/kg (ns).

The effects of PRA on startle magnitude were balanced across strains, as above, using values for 0.03 mg/kg PRA and vehicle (SD: vehicle = 106.4 (21.1), PRA 0.03 mg/kg=105.6 (15.7), PRA 0.06 mg/kg = 109.5 (25.0), n = 9; LE: vehicle = 197.5 (42.5), PRA 0.03 mg/kg = 225.2 (21.0), PRA 0.06 mg/kg = 231.0 (40.0), n=9). ANOVA of P-ALONE amplitude in these rats revealed a significant effect of strain (F=8.40, df 1,16, p <0.02), but not dose (F<1), and no strain × dose interaction (F<1). ANOVA of %PPI in these subgroups confirmed the key results: a significant effect of strain (F=35.79, df 1,16, p<0.0001) and dose (F=7.80, df 1,16, p<0.002), but no strain × dose interaction (F=1.18, df 1,16, ns). Importantly, the magnitude of the PRA effect (%PPI$_{vehicle}$-%PPI$_{PRA}$) was not significantly greater in SD than LE rats for either 0.03 mg/kg or 0.06 mg/kg (both ns).

For the higher PRA dose range (0.1 - 1.0 mg/kg), ANOVA of %PPI revealed a trend towards a strain effect (F=3.00, df 1,22, 0.05<p<0.1), and a significant effect of dose (F=20.80, df 2,44, p<0.0001), but no significant strain × dose interaction (F<1). The magnitude of the PRA effect (%PPI$_{vehicle}$-%PPI$_{PRA}$) was not significantly greater in SD than LE rats for either 0.1 mg/kg (F=1.26, df 1,22, ns), or 1.0 mg/kg (F<1) of PRA. Post-hoc analysis in SD rats revealed that %PPI was significantly reduced by 0.1 mg/kg (p<0.005) and 1.0 mg/kg (p< 0.0001) PRA relative to vehicle; in LE rats, these effects reached significance only for 1.0 mg/kg PRA (p<0.005) (Fig. 6.2B).

Based on the strong trend towards strain differences in PPI, subgroups were balanced for vehicle PPI levels across strains (mean (SEM) vehicle PPI: SD (n=6): 69.4 (1.4); LE (n=6): 68.8 (1.9)). The subgroups were also comparable with respect to baseline (vehicle) startle magnitude (mean (SEM): SD: 201.0 (13.2); LE: 199.7 (74.2)). ANOVA

of %PPI in these subgroups revealed a significant main effect of PRA dose (F=11.26, df 2,20, p<0.001), but neither an effect of strain (F<1), nor a strain × dose interaction (F<1). The magnitude of the PRA effect on PPI (%PPI$_{vehicle}$-%PPI$_{PRA}$) revealed a trend towards greater PPI PRA sensitivity in LE than SD rats at 0.1 mg/kg (F=4.44, df 1,10, 0.05<p<0.1), but not at 1.0 mg/kg (F<1).

ANOVA of startle magnitude on P-ALONE trials revealed a trend towards a strain effect (F=4.07, df 1,22, 0.05<p<0.1), a significant effect for dose (F=4.62, df 2,44, p<0.02), but no strain × dose interaction (F=2.12, df 2,44, ns) (Fig. 6.2B, inset). The magnitude of PRA-induced startle suppression in SD rats made it impossible to form subgroups balanced for PRA effects on this measure. However, simple regression showed that this effect contributed less than 1% of the variance to the observed PRA effect on PPI for SD vs. LE rats for the highest dose of PRA (1.0 mg/kg; ns). For 0.1 mg/kg of PRA, this effect contributed to less than 8% of the observed PRA effect on PPI (ns) in LE rats, and to less than 20% in SD rats (ns).

NOSTIM levels for both low and high PRA dose range were < 1% of pulse alone amplitudes. For low dose ranges, ANOVA revealed a trend towards a strain effect (F=4.08 df 1,22, p<0.1), but no effect of dose (F<1) or dose × strain interaction (F=1.73, df 2,44, ns). For the high dose ranges, ANOVA revealed a significant main effect of strain (F=5.77 df 1,22, p<0.05), but not dose (F=1.84 df 2,44, ns), and no PRA dose × strain interaction (F<1). Simple regression showed that PRA effects on NOSTIM did not contribute a significant proportion of the variance to the observed PRA effect on PPI for

any of the rat strains and PRA doses (all regression weights ns). Thus, PRA effects on NOSTIM levels cannot account for the observed PRA effects on PPI.

Figure 6.2: Effects of 0.03 - 0.06 mg/kg (A) or 0.1-1.0 mg/kg (B) PRA on PPI and startle magnitude (insets) in SD and LE rats. (A) ANOVA of PPI revealed significant effects of strain (SD>LE) and PRA, but no strain × PRA interaction. Thus, unlike APO (Fig. 6.1A) and QUIN (Fig. 6.1B), SD vs. LE rats did not differ in the PPI-disruptive effects of PRA. This was also true in subgroups balanced for basal PPI levels and PRA effects on startle magnitude. (B) As with the lower dose range of PRA (Fig. 6.2A), SD vs. LE rats did not differ in the PPI-disruptive effects of PRA. Again, this was true in subgroups balanced for basal PPI levels. The magnitude of startle suppression in SD rats made it impossible to form subgroups balanced for PRA effects on this measure. However, a simple regression showed that this effect did not contribute a significant proportion of variance to the observed PRA effects on PPI in any of the strains and PRA doses tested (regression weights n.s.). [#] p<0.1, * p<0.05, ** p<0.005, *** p<0.0001.

4. Effects of L741,626: The D2 receptor antagonist L741,626 was used to assess the contributions of D2 receptor activation to the PPI-disruptive effects of APO and PRA in SD rats.

Studies first tested the effects of pretreatment with L741,626 (0, 1, 3, 10 mg/kg) on the PPI-disruptive effects of 0.5 mg/kg APO. ANOVA revealed significant main effects of APO (F=71.11, df 1,12, p<0.0001), and L741,626 (F=10.70, df 3,12, p<0.002), and a significant APO × L741,626 dose interaction (F=24.96, df 3,12, p<0.0001). Post-hoc analysis revealed that APO significantly reduced %PPI in rats treated with 0 mg/kg L741,626 (p<0.005). After APO treatment, PPI was significantly different between groups treated with 0 mg/kg L741,626 and each active dose of L741,626 (vehicle vs. 1 mg/kg (p<0.002); vehicle vs. 3 mg/kg (p<0.0001); vehicle vs. 10 mg/kg (p<0.0001)) with greater %PPI values in rats treated with L741,626 (Fig. 6.3A).

Studies next tested the effects of pretreatment with L741,626 (0, 1, 3, 10 mg/kg) on the PPI-disruptive effects of PRA (0 vs. 1 mg/kg). ANOVA of PPI revealed significant main effects of PRA (F=15.50, df 1,28, p<0.001), and L741,626 (F=4.56, df 1,28, p<0.02), and a significant PRA × L741,626 dose interaction (F=3.98, df 3,28, p<0.02). Post-hoc analysis revealed that PRA significantly disrupted PPI in rats treated with 0 mg/kg L741,626 (p<0.005). These effects were significantly opposed by 3 mg/kg and 10 mg/kg (p<0.02) of L741,626, but not by 1 mg/kg of L741,626 (p>0.6) (Fig. 6.3C).

Conceivably, the ability of the lowest dose of L741,626 to prevent APO- but not PRA-induced PPI deficits may have reflected greater range produced by the more robust

PPI-disruptive effects of this dose of APO. To assess this possibility, we repeated the experiment, using a lower dose of APO (0 vs. 0.1 mg/kg) and the critical doses of L741,626 (0 vs. 1mg/kg). ANOVA of PPI revealed a significant main effect of APO (F=16.42, df 1,14, p<0.002), no significant main effect of L741,626 (F=1.86, df 1,14, ns), but importantly, a significant APO × L741,626 dose interaction (F=16.33, df 1,14, p<0.002). APO significantly reduced %PPI in rats treated with 0 mg/kg of L741,626 (p<0.005), and this effect was significantly opposed by 1 mg/kg L741,626 (p<0.001) (Fig. 6.3B).

While not described above, in all studies with L741,626 vs. APO or PRA, drug effects on startle magnitude (see Figure 6.3) and NO STIM levels could not account for the observed patterns of PPI.

Figure 6.3: Effects of the D2 receptor antagonist L741,626 on PPI deficits induced by 0.5 mg/kg APO (A), 0.1 mg/kg APO (B), or 1 mg/kg PRA (C) and startle magnitude (insets) in SD rats. (A) 0, 1, 3, and 10 mg/kg of L741,626 were tested against a standard dose of APO of 0.5 mg/kg. APO significantly reduced %PPI in rats treated with 0 mg/kg of L741,626, and this effect was significantly opposed by each active L741,626 dose. ANOVA of startle magnitude on P-ALONE trials revealed no effect APO, and L741,626, and no APO × L741,626 interaction effect. (B) 0 and 1 mg/kg of L741,626 were tested against a low dose of APO (0.1 mg/kg), selected to match the magnitude of PPI disruption achieved by 1 mg/kg of PRA (see (C)). Again, APO significantly reduced PPI, and this effect was significantly opposed by 1 mg/kg L746,626. ANOVA of startle magnitude on P-ALONE trials revealed no main effect of APO, and L741,626, and no APO × L741,626 interaction. (C) 0, 1, 3, and 10 mg/kg of L741,626 were tested against 1 mg/kg of PRA. PRA significantly disrupted %PPI in rats treated with 0 mg/kg L741,626. This effect was significantly opposed by the 3 mg/kg and 10 mg/kg doses of L741,626, but *not* by the 1 mg/kg dose ($p > 0.6$; Fig. 6.3C). ANOVA of startle magnitude on P-ALONE trials revealed a significant main effect of PRA, reflecting reduced startle magnitude in PRA-treated rats. No effect of L741,626, and no PRA × L741,626 interaction effect were detected. Significance levels derived from post-hoc comparisons between vehicle and DA agonist treatment in rats treated with 0 mg/kg L741,626 are denoted with * symbols. Significance levels derived from comparisons between vehicle and L741,626 pretreatment in rats treated with DA agonists are denoted with [&] symbols. ** $p < 0.005$; [&] $p < 0.05$; [&&] $p < 0.005$; [&&&] $p < 0.0001$.
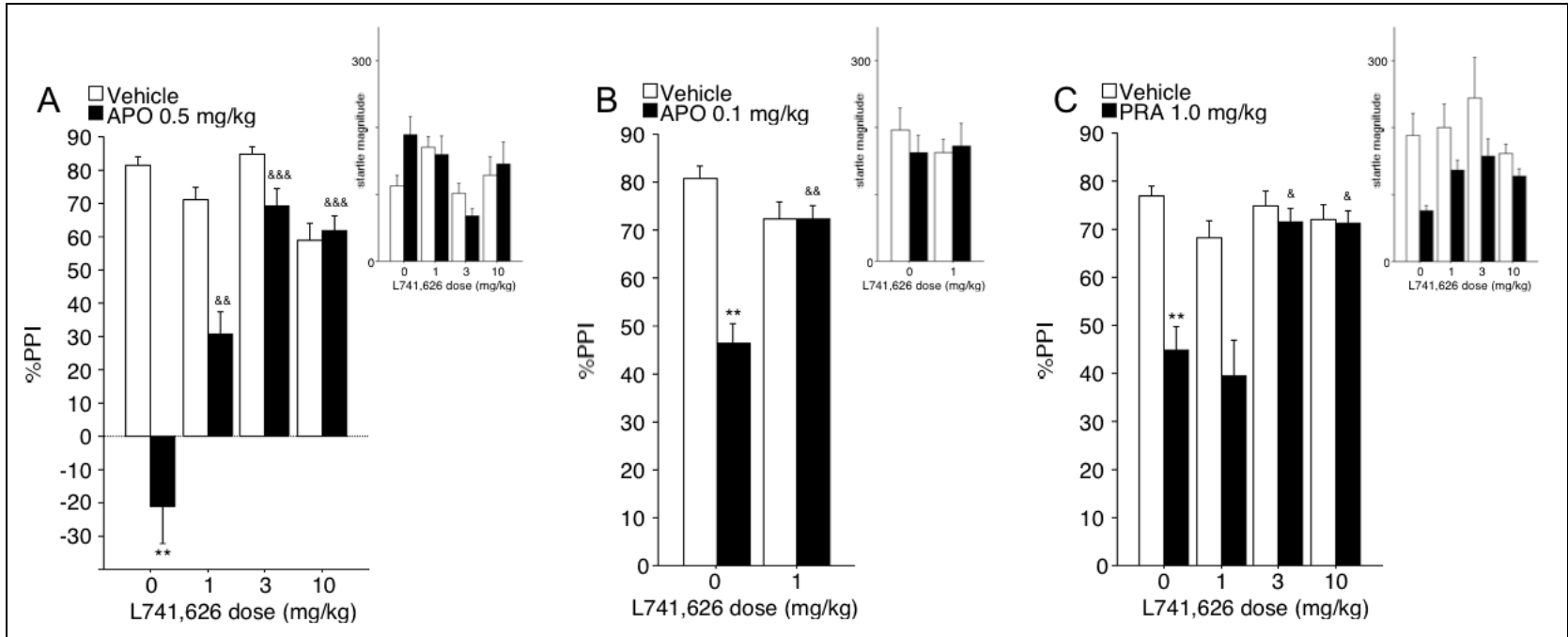
E. Discussion:

We previously reported greater sensitivity of SD than LE rats to the PPI-disruptive effects of systemically administered DAergic agonists such as APO, AMPH, or QUIN (Swerdlow et al., 2001b, 2003b, 2004a, 2004b, 2004c; Weber and Swerdlow, 2008). The strain differences in PPI-APO and -QUIN sensitivity were confirmed in this study and extended by detailed analyses showing that these strain differences could not be attributed to strain differences with respect to 1) baseline (vehicle) PPI, 2) baseline (vehicle) startle magnitude; 3) drug effects on startle magnitude, or 4) drug effects on NOSTIM levels.

Here we report for the first time the PPI-disruptive effect of the preferential D3 receptor agonist, PRA. The disruption of PPI in rats by PRA confirms earlier reports with other preferential D3 agonists such as ropinirole, 7-OH-DPAT, or PD128,907 (Caine et al., 1995; Swerdlow et al., 1998a; Varty and Higgins, 1998; Zhang et al., 2007). With the exception of the Islands of Calleja, the highest levels of D3 receptor densities have been detected in the nucleus accumbens (NAC) in both rodents and humans (cf. Sokoloff et al., 2006). The disruption of PPI by PRA in two separate rat strains is thus consistent with a prominent role of the NAC in the regulation of PPI (Swerdlow et al., 1986; cf. Swerdlow et al., 2001a).

Importantly, in the present study, we demonstrated for the first time that heritable strain differences in the PPI-disruptive effects of the mixed D1/D2-like agonist, APO, and the mixed D3/D2 agonist, QUIN, do not extend to the preferential D3 agonist PRA. In contrast, sensitivity to the PPI disruptive effects of PRA was comparable between SD

and LE rats, and this finding persisted in careful analyses controlling for 1) baseline (vehicle) PPI, 2) baseline (vehicle) startle magnitude; 3) PRA-effects on startle magnitude, or 4) PRA-effects on NOSTIM activity. In instances where trends were detected for strain differences in PRA sensitivity, LE rats tended to be more sensitive to the PPI-disruptive effects of PRA, compared to SD rats.

Conceivably, relatively reduced PPI in LE rats after vehicle treatment might contribute to the blunted impact of APO and QUIN on PPI, via a "floor effect". However, a floor effect cannot explain the present findings, because in subsets of rats matched for baseline PPI, or following analyses of covariance to control for sources of variance related to baseline PPI, SD rats were still more sensitive to the PPI-disruptive effects of APO and QUIN. Differential APO and QUIN sensitivities of startle magnitude might also conceivably contribute to SD vs. LE difference in PPI APO and QUIN sensitivity, but the present data also do not support such an interpretation because these PPI differences were evident even among "matched" subsets of SD and LE rats that exhibited comparable APO and QUIN effects on startle. Finally, it could be argued that differential APO and QUIN sensitivities on generalized motor activity (NOSTIM levels) may contribute to the observed strain differences with respect to the APO PPI-sensitivity. However, this explanation was also ruled out for both drugs, based on regression analyses.

Since the discovery and cloning of the D3 receptor (Sokoloff et al., 1990), *in vivo* experiments addressing D3 receptor activation and inhibition have been hampered by several factors. First, commercially available, preferential D3 receptor ligands also have significant affinity for D2 receptors (Luedtke and Mach, 2003; Sokoloff et al., 2006)

suggesting that higher doses of these compounds bind to both D3 and D2 receptors. Pramipexole represents one of the more preferential D3 agonists, with an *in vitro* D3:D2 preference of 7:1 (Piercey et al., 1996, Svensson et al., 1994a) relative to the high affinity state of the D2 receptor. Millan and coworkers (2002) have determined the D3:D2 preference of pramipexole to be 90:1 relative to the short isoform of the human receptor (D2S), and 160:1 relative to the long isoform (D2L; Millan et al., 2002). Second, D3 and D2 receptors appear to form domain-swapping heterodimers (cf. Maggio et al., 2003). Conceivably, this leads to significant challenges of dissecting D3- vs. D2-mediated effects in rats. Despite these challenges, however, recent evidence from *in vivo* studies in rats suggests that PRA doses of approximately 0.1 mg/kg or less, predominantly lead to D3 receptor associated effects, in the relative absence of D2-receptor associated effects (Collins et al., 2007).

One strategy to approach these challenges is to compare the effects of drugs characterized by a range of DA receptor-subtype affinities. To determine which DA receptor subtypes contribute to the SD vs. LE strain differences in PPI sensitivity, we compared drugs with prominent affinity for D1/D2-like receptors (APO), D3/D2 receptors (QUIN) and D3 receptors (PRA). While none of the drugs are receptor-specific in their binding profiles, the D3:D2 binding ratio for QUIN is approximately 10-fold lower than that for PRA (Piercey et al., 1996). Thus, evidence for SD > LE PPI sensitivity for QUIN but not PRA further argues that this phenotype reflects heritable differences in the sensitivity of D2 but not D3 receptors.

This view was supported by studies in which the effects of APO and PRA were tested against a dose-range of the D2 receptor antagonist L741,626 in SD rats. An alternative strategy would have been to test the effects of APO and PRA against highly preferential D3 receptor antagonists such as SB-277011A and S33084, but such well-characterized D3 antagonists (cf. Joyce and Millan, 2005) remain difficult to obtain. L741,626 is among the most selective commercially available D2 receptor antagonist (Joyce and Millan, 2005, Millan et al., 2000c). Nevertheless, its D2:D3 preference ratio is modest and has been estimated as 4:1 for native rat receptors (Cussac et al., 2003). This suggests that at higher doses, L741,626 is likely to lose its preferential D2 inhibition and is likely to inhibit D2 and D3 receptors. In line with this, at the highest doses tested (3-10 mg/kg), L741,626 reversed PPI deficits induced by APO and by PRA. Nevertheless, the lowest dose of L741,626 had no detectable impact on PRA-induced PPI deficits, but significantly opposed the PPI-disruptive effects of both 0.1 and 0.5 mg/kg APO. The lower APO dose was selected to disrupt PPI by an amount comparable to that produced by PRA. Thus, under conditions of "matched PPI deficits", a low dose of L741,626 fully reversed APO-induced PPI deficits, whereas PRA-induced PPI deficits were completely unaffected. These data argue against a major involvement of D2 receptor activation in the induction of PRA-induced PPI deficits, and by extension, suggest that the PPI-disruption induced by PRA is predominantly due to D3 activation. In addition, L741,626 has substantially higher binding ratios for D2 than for D1 (~200), D4 (~80), or D5 (~160) receptors (Millan et al., 2000). While we cannot exclude that cooperation between D2 receptors and these other receptor subtypes may occur, these data indicate that the

prevention of APO-induced PPI deficits by the low dose of L741,626 is unlikely due to blockade of D1, D4, or D5 receptors, independent of its antagonism of D2 receptors.

Even though PRA displays preferential binding to D3 receptors relative to all other receptor types, it is not possible to entirely exclude an involvement of a small number of other receptor types in PPI deficits induced by PRA, including the D4 receptor, and perhaps the α_{2B} adrenergic and 5-HT1$_A$ serotonergic receptors. First, the *in vitro* binding ratio of PRA to D3 vs. D4 receptors has been approximated as 12:1 (Millan et al., 2002) or 17:1 (Piercey et al., 1996). This indicates that D4 receptor activation may play some role after treatment with the highest PRA doses tested. Nevertheless, it would be difficult to attribute the lack of SD > LE sensitivity to disruption of PPI by PRA to a major D4 receptor involvement for the lower PRA doses tested. Second, Millan et al. (2002) reported relatively low preferential binding rates in the range of 60:1 for PRA to the D3 receptor relative to the α_{2B} and 5-HT1$_A$ receptors. In contrast, Piercey et al. (1996) reported values of > 200:1 for α_2 and >1000 for 5-HT1$_A$ receptors. Again, if anything, this may indicate an involvement of these receptor types in PPI deficits induced by the highest doses of PRA, but less likely so for the lowest doses of PRA tested. For all other receptors tested, including DA D1 and D5, preferential binding ratios to the D3 receptor have been assessed as 150:1 or greater by both studies (Millan et al., 2002, Piercey et al., 1996). Nevertheless, it is important to acknowledge that none of these pharmacological tools have absolute receptor specificity, and behavioral responses to these drugs cannot be attributed with certainty to any single receptor or receptor subtype. Perhaps the most conservative conclusion is that, based on the convergent evidence from the present

studies, the D2 receptor does not appear to play a prominent role in the PPI-disruptive effects of PRA; by extension, the heritable differences in sensitivity to the PPI-disruptive effects of D2 stimulation are not manifested in a differential sensitivity to PRA.

Our previous studies have determined that the SD vs. LE differences in PPI "disruptability" by DA agonists reflects processes localized within the NAC (Swerdlow et al., 2007), associated with NAC DA-stimulated GTPγS binding (Swerdlow et al., 2006a), CREB phosphorylation (Saint Marie et al., 2007), suppression of *c-fos* expression (Saint Marie et al., 2006) and specific patterns of gene activation (Shilling et al., 2008). The present findings suggest that the heritable behavioral phenotype reflects differential sensitivity of D2 but not D3 receptors. These findings further suggest the testable hypothesis that the D2- and D3-regulation of PPI are mediated via mechanisms that differ at the levels of NAC DA-linked signal transduction and the genes associated with this process.

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CHAPTER 7:

The effects of the dopamine D2 agonist sumanirole on prepulse inhibition in rats

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A. Abstract:

Dopamine agonists reduce prepulse inhibition (PPI) of startle in rats. While it is used to predict antipsychotic efficacy, the specific receptor subtypes mediating this effect of dopamine agonists remains unclear. We characterized the effects of sumanirole, a highly selective D2 agonist, on PPI in rats. Sumanirole decreased PPI at 60-120 ms prepulse intervals, and increased PPI at 10-20 ms intervals. PPI deficits were antagonized by low doses of the preferential D2 antagonist L741626, supporting a D2 mechanism of action. Sumanirole is a valuable tool for parsing the role of dopamine receptor subtypes in the regulation of PPI.

B. Introduction:

The ability of compounds to prevent the disruption of prepulse inhibition (PPI) by non-selective dopamine (DA) agonists like apomorphine (APO) in rats is widely used to identify compounds with antipsychotic properties (cf. Swerdlow et al., 2008b). In the absence of both receptor-specific knock-out rats and subtype-selective DA receptor

compounds, however, relatively little is known about the role of DA receptor subtypes in regulating these PPI deficits in this species. For example, it is difficult to distinguish DA D2- vs. D3-receptor linked effects in these models. *In vivo* screens have utilized the ability to oppose PPI deficits induced by pramipexole (PRA) or PD128907 as a basis for identifying D3-preferential receptor antagonists for clinical applications (Weber et al., 2009b; Zhang et al., 2007). In these studies, greater sensitivity to oppose PPI deficits induced by D3 agonists than by non-specific DA agonists is used to suggest D3-preferential receptor blockade. The sensitivity of this *in vivo* assay to distinguish antagonists with primary D3 vs. D2-linked mechanisms would be enhanced by highly D2-preferential agonists that reliably disrupt PPI. Here, we assessed the PPI-disruptive effects of sumanirole (SUM), a novel D2-selective agonist (Heier et al., 1997).

Based on *in vitro* receptor binding, the D2 affinity of SUM exceeds that of other DA receptor subtypes by over 200-fold (cf. de Paulis, 2003; McCall et al., 2005; Wuts et al., 2002); accordingly, studies have used SUM to parse D2 vs. D3-receptor effects in assays of hypothermia and drug discrimination (Achat-Mendes et al., 2009; Collins et al., 2007; Koffarnus et al., 2008). To our knowledge, no study has evaluated SUM in animal models for schizophrenia.

C. Methods:

1. Animals: Adult male Sprague Dawley rats (n = 49; 225-250 g; Harlan, Livermore, CA) were handled 1d after arrival, housed in groups of 2-3, and maintained on a reversed light/dark schedule with water and food *ad libitum*. Testing occurred during

the dark phase. Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the UCSD Animal Subjects Committee (protocol #S01221).

2. Drugs: Ascorbic acid and APO hydrochloride hemihydrate (Sigma; St. Louis, MO), PRA hydrochloride (TRC; North York, Canada), and L741626 (Tocris; Ellisville, MO) were used. Sumanirole maleate was supplied by the NIMH Chemical Synthesis and Drug Supply Program. SUM and PRA were dissolved in saline, APO in 0.01 % ascorbate/saline, and L741626 in 0.03% lactic acid/water (w/v; pH ≥5 with NaOH). Doses (mg/kg salt) were administered subcutaneously in 1 ml/kg. L741626 was administered 30 min prior to SUM. SUM was administered immediately before the "time course" experiment, and 15 min prior to testing in all subsequent experiments. Based on previous studies (Weber et al., 2008a, 2009b), PRA and APO were administered 15 and 0 min before testing, respectively.

3. Apparatus: Startle chambers (SD Instruments, San Diego, CA) (Weber et al., 2008a, 2009b) recorded 100 1-ms readings beginning at stimulus onset. Startle magnitude was the average of these 100 readings.

4. Testing: All studies used a continuous 70 dB(A) background noise (all sound calibrated on the "A" scale); after 5 min, trials were presented in pseudorandom order.

Approximately 7 days after arrival, rats completed a brief startle test; based on the results, rats were assigned to drug dose groups with matched baseline %PPI (Weber et al., 2008a, 2009b). Inter-test intervals were 3 - 7 days. Variable inter-trial intervals averaged 15s. Interspersed between active trials were trials in which no stimulus was presented, but cage displacement was measured (NOSTIM trials).

a. Protocol 1: (65 min) established the time course of SUM effects on PPI, using a within-subject balanced dose order design. After the acclimation period, rats were exposed to six 10 min blocks consisting of 5 min of startle trials followed by 5 min without trials. Blocks began with a P120 stimulus (a 40 ms - 120 dB noise burst; not included in the calculation of PPI), followed by a mixture of 6 P120, 6 PP12+P120 (P120 preceded 100 ms (onset-to-onset) by a 20 ms noise burst of 12 dB above background), and 4 PP12 stimuli (PP12 pulses *not* followed by P120).

b. Protocol 2: (18.25 min) tested the dose-response properties of SUM using a between-subject design, and the effects of SUM vs. L741626 using a mixed-model, balanced dose-order design with L741626 as the between- and SUM as the within-factor. Twenty-three rats were first tested in the dose-response experiment, then redistributed to dose groups according to baseline PPI and SUM drug group. After a "washout" period of 5 d, these rats were tested in the SUM × L741626 experiment. The session began with 4 and ended with 3 consecutive P120 trials; between these trials were 2 blocks, each consisting of 8 P120 trials, 5 PP5+P120 trials, 5 PP10+P120 trials, and 5 PP15dB+P120

trials, i.e. trials in which the P120 was preceded 100 ms (onset to onset) by a prepulse of 20 ms duration and an intensity of either 5, 10, or 15 dB above background, respectively.

c. Protocol 3: (15.5 min) evaluated the effects of SUM, APO and PRA at 10 - 120 ms prepulse intervals. Twenty rats first used for the SUM experiment (above) were redistributed into balanced dose groups; after a "washout" period of 16 d, these rats were used in the APO/PRA experiment. The session began and ended with 3 consecutive P120 trials. Between these trials were 6 P120, and 6 PP10ms+ P120, 6 PP20ms+ P120, 6 PP30ms+P120, 6 PP60ms+ P120, or 6 PP120ms+ P120 trials, i.e. trials in which the P120 was preceded 10, 20, 30, 60, or 120 ms by a 5 ms prepulse that was 15 dB above background.

5. Data analysis: PPI was defined as 100-[(startle magnitude on prepulse trials / startle magnitude on P120 trials) × 100], and was analyzed by ANOVAs. Post-hoc comparisons used ANOVAs or Fisher's PLSD. Data were collapsed across prepulse intensities and blocks (protocol 2). Alpha was 0.05.

D. Results:

1. Time course study: This study was conducted to identify adequate pretreatment times for SUM (0 vs. 3.0 mg/kg). ANOVA of %PPI revealed a main effect of SUM dose (F = 7.1; df 1,5, $p<0.05$), but no significant effects of time (F = 1.6; df 1,5;

n.s.), or time × dose interaction (F < 1). Based on inspection of the data (Fig. 7.1A), subsequent studies utilized a SUM pretreatment interval of 15 min.

2. Dose-response study: The effects of SUM (0, 0.3, 1.0, 3.0 mg/kg) were tested next. ANOVA of %PPI revealed a main effect of SUM dose (F=3.1; df 3,19; p=0.05). Post-hoc tests revealed that each active dose of SUM significantly reduced PPI relative to the vehicle condition, (p<0.05 for 0.3 and 1.0 mg/kg; p<0.005 for 3.0 mg/kg of SUM) (Fig. 7.1B).

3. L741626 study: The effects of SUM (0, 3.0 mg/kg) on PPI were tested after pretreatment with the D2-preferential antagonist L741626 (0, 0.3, 0.6 mg/kg). ANOVA of %PPI revealed a significant effects of SUM (F=16.4; df 1,20; p<0.001) and a SUM × L741626 interaction (F=9.5; df 2,20; p<0.005). No other effects were significant. Post-hoc analyses revealed that SUM decreased %PPI in rats pretreated with 0 mg/kg of L741626 (p<0.005), and this effect was opposed in animals pretreated with 0.3 (p<0.05) or 0.6 (p<0.0005) mg/kg of L741626 (Fig. 7.1C).

4. Interval study: The effects of SUM (0, 3.0 mg/kg) were tested at varying prepulse intervals. ANOVA of % PPI revealed significant effects of prepulse interval (F=18.6; df 4,72; p<0.0001), and a significant interaction of SUM dose × prepulse interval (F=7.5; df 4,72; p<0.0001). Post-hoc analyses revealed significant PPI-

increasing effects of SUM at short prepulse intervals (10 - 20 ms; p<0.05) and significant

PPI-*decreasing* effects of SUM at long prepulse intervals (60 ms and 120 ms; p<0.0001)

(Fig. 7.1D).

We next compared these effects of SUM to those of APO and PRA, using doses that disrupt long interval PPI by magnitudes comparable to that produced by 3 mg/kg of SUM. ANOVA of % PPI revealed significant effects of prepulse interval (F=24.5; df 4,68; p<0.0001) and interval × drug interaction (F=2.1; df 4,68; p<0.05). Post-hoc analyses revealed no PPI-enhancing effects of PRA or APO at short (10 - 20 ms) prepulse intervals, but PPI-reducing effects of both PRA (p<0.001) and APO (p<0.005) at long (60 - 120 ms) prepulse intervals (Fig. 7.1E).

In all protocols, SUM effects on startle magnitude were not statistically significant; when non-significant trends towards a SUM effect on startle magnitude were observed, simple regression analyses revealed that these trends could not account for SUM effects on PPI. All main or interaction effects of SUM, APO or PRA on NOSTIM or prepulse only activity (protocol 1) were not statistically significant. Startle magnitude for APO and PRA followed previously published patterns (Table 7.1; Weber et al., 2008a, 2009b).

Figure 7.1: The effects of SUM (A-D), and APO or PRA (E) on PPI. **(A)** Time course of SUM effects. **(B)** Dose-response effects of SUM. **(C)** L741626 antagonized SUM-induced PPI deficits**. (D)** SUM *decreased* PPI at long prepulse intervals, but *increased* PPI at short prepulse intervals. **(E)** APO and PRA decreased PPI at long prepulse intervals, but PPI at short prepulse intervals was unaffected. * denotes significant differences for treatment with SUM, APO, or PRA vs. vehicle, [&] denotes significant antagonism of the SUM-induced PPI deficit by L741626; *·[&] p<0.05; ** p<0.005; ***·[&&&] p<0.0005.

Table 7.1: Effects of SUM, APO, or PRA on startle magnitude. SUM effects on startle magnitude were independent from SUM effects on PPI (see results).

EXPERIMENT	Drug(s)	Startle Magnitude Mean (SEM)
1. Time course	Vehicle:	400 (79)
	SUM (3 mg/kg):	237 (49)
2. SUM Dose-response	Vehicle:	187 (6)
	SUM (0.3 mg/kg):	213 (54)
	SUM (1.0 mg/kg):	270 (65)
	SUM (3.0 mg/kg):	242 (31)
3. L741626 × SUM	Vehicle/Vehicle:	182 (60)
	Vehicle/L741626 (0.3 mg/kg):	206 (43)
	Vehicle/L741626 (0.6 mg/kg):	267 (34)
	SUM/Vehicle:	174 (34)
	SUM/L741626 (0.3 mg/kg):	226 (35)
	SUM/L741626 (0.6 mg/kg):	290 (32)
4. Prepulse Intervals	Vehicle:	259 (45)
	SUM (3.0 mg/kg):	243 (33)
	Vehicle:	246 (32)
	APO (0.1 mg/kg):	161 (33)
	PRA (0.3 mg/kg):	140 (26)

E. Discussion:

In this study, SUM disrupted PPI under testing conditions widely used in published studies that assessed antipsychotic potency (cf. Swerdlow et al., 2008b) in a dose range linked to D2 receptor activation in rats (Collins et al., 2007; Koffarnus et al., 2008). While having marked preferences for D2 receptors over all other types of DA receptor subtypes (>200 fold) – SUM has only a moderate (~8 fold) binding preference for the D2 vs. 5-HT$_{1A}$ receptors (cf. de Paulis 2003; McCall et al., 2005). Hence, it was critical to test SUM against the preferential D2 antagonist L741626, a compound with ~10 fold binding preference for D2 receptors relative to D3 receptors, and 80-200 fold preferences relative to all other DA receptors, and non-DA receptors, including the 5-HT$_{1a}$ receptor (Cussac et al., 2000; Millan et al., 2000c, 2004b). The fact that *very low* doses of L741626 favoring D2 receptor blockade fully antagonized SUM-induced PPI deficits strongly supports a D2 (rather than a D3 and/or 5HT1a) receptor linked mechanism of action of SUM in measures of PPI.

SUM both *decreased* PPI at long prepulse intervals and *increased* PPI at low prepulse intervals; at doses that generated a comparable degree of long-interval PPI-reduction, the non-selective DA agonist APO, and the D3 preferential agonist PRA did not increase short-interval PPI. Importantly, studies testing higher doses of APO (0.5 mg/kg), or using PRA in more sensitive within-subjects designs both have detected increased short-interval PPI (Swerdlow et al., 2004a, 2009a), suggesting that the differences vs. SUM detected here are ones of degree, i.e. compared to PRA and APO, SUM has a more potent effect on the substrate responsible for the increased short interval PPI.

The present findings extend previous studies with non-selective DA agonists like APO and quinpirole (cf. Swerdlow et al., 2008b; Millan et al., 2002; Weber et al., 2008a, 2009b; Zhang et al., 2007). While these studies showed that co-activation of D2-receptors with either D1, and/or D3 receptors potently disrupts PPI in rats, the present findings demonstrate that D2 receptor activation is sufficient to disrupt PPI deficits in rats, even at SUM doses that would be predicted to have *no appreciable co-activation of either D1 and/or D3 receptors*. Such a selective D2-receptor linked mechanism of action of SUM on PPI will be valuable in parsing the neurobiological basis of antipsychotic-like effects in rodent PPI models. In particular, SUM may be valuable in interpreting findings in *in vivo* assays that use PPI to detect D3-preferential antagonists (Weber et al., 2009b; Zhang et al., 2007) or other novel antipsychotics (e.g. Wadenberg et al., 2000), and in clarifying the PPI-regulatory role of intracellular signaling pathways linked to D2 vs. D3 receptor activation (Chang et al., 2009; Saint Marie et al., 2007).

F. Acknowledgements:

CHAPTER 8:

The effects of pramipexole on prepulse inhibition and locomotor

activity in C57BL/6J mice

Wei-li Chang, Mark A. Geyer, Mahalah R. Buell, Martin Weber, Neal R. Swerdlow

A. Abstract:

Pramipexole (PRA) is a preferential D3R agonist that in rats and humans modifies prepulse inhibition (PPI) of the acoustic startle reflex, an operational measure of sensorimotor gating. The ability to use similar PPI measures across species, and the relative ease of genetic manipulations in mice, suggests that molecular studies of the D3R regulation of sensorimotor gating might be best pursued in mice. Here, we evaluate the effects of PRA on PPI and locomotion in C57BL/6J mice, the background strain for many gene knockout mouse models. Male C57BL/6J mice were tested for PPI and locomotor activity after injection of PRA. No significant effects of PRA on PPI were seen at any dose (0.1-10.0 mg/kg), but a significant reduction in startle magnitude was observed after 10 mg/kg PRA. In contrast, the D1/2 agonist, apomorphine (5 mg/kg) significantly reduced PPI in these mice. At doses of PRA that did not alter startle magnitude (0.3, 1.0, 3.0 mg/kg), significant decreases in the amount of locomotor and investigatory behavior were observed. Distinct from findings in rats and humans, it appears that either: 1) PRA does not activate D3Rs in C57BL/6J mice, or 2) D3R agonists are not sufficient to alter PPI in this mouse strain.

B. Introduction:

The study of dopamine (DA) D3 receptors (D3R) may shed new light on the pathogenesis and therapy of several neuropsychiatric disorders, but, due to the homology between D3 and D2 receptors, there are only a small number of well-characterized specific D3 and D2 agonists and antagonists. Pramipexole is a non-ergot dopamine D2/D3 receptor full agonist that has been reported to have a D3:D2 binding preference ranging from 7:1 to 160:1 *in vitro* (Millan et al., 2002; Piercey et al., 1996; Svensson et al., 1994a). In rats, pramipexole is a preferential D3 receptor agonist that reduces prepulse inhibition (PPI) of acoustic startle, an operational measure of sensorimotor gating (Weber et al., 2008a, 2009b). In humans, pramipexole significantly increases PPI (Talledo et al., 2009), an effect observed with other DA receptor agonists in some populations (Bitsios et al., 2005). Though various *in vitro* and pharmacological techniques have been employed to assess the receptor specificity of pramipexole, *in vivo* molecular manipulations would provide definitive evidence of D3 vs. D2 contributions to effects of pramipexole on sensorimotor gating. Such molecular mechanisms might also shed light on the molecular basis for the D3 regulation of PPI, which in humans appears to be linked to the D3 receptor Ser9Gly polymorphism (Roussos et al., 2008a).

In mice, dopamine D3 receptor knock-out models have been informative about receptor subtype contributions for the actions of many dopaminergic drugs (Carta et al., 2000; Doherty et al., 2008; Glickstein and Schmauss, 2004; Harrison and Nobrega, 2009; Karasinska et al., 2005; Le Foll et al., 2002; Leggio et al., 2008; McNamara et al., 2006; Ralph et al., 1999; Risbrough et al., 2006; Schmauss, 2000; Siuciak and Fujiwara, 2004;

Zhou et al., 2007). Pramipexole has been found to alter nocturnal locomotion, alter operant responding (Lehr, 2002), induce hypothermia (Maj et al., 1997), and decrease the duration of immobility in the forced swim test (Kitagawa et al., 2009; Siuciak and Fujiwara, 2004). The ability to use similar PPI measures across species and the relative ease of genetic manipulations in mice makes them an attractive animal model to further study the role of D3 receptor activation in the regulation of sensorimotor gating. To date, there have not been any published reports of pramipexole effects on PPI in any strain of mice. Previous studies of PRA effects on locomotor activity have had mixed results with different strains of mice. The current report will describe the effects of pramipexole on PPI and locomotor activity in C57BL/6J mice, a common background strain for knockout mice.

C. Methods:

1. Subjects*:* Male C57BL/6J mice ($n = 76$) between 7 and 12 weeks of age were obtained from Jackson Labs (Bar Harbor, ME) and housed at a vivarium at the University of California San Diego (UCSD), an AAALAC-approved animal facility that meets Federal and State requirements for care and treatment of laboratory animals. Mice were allowed to acclimate for approximately 1 week after arrival. All mice were housed n = 4 per cage, in a climate-controlled room with a reversed light cycle (lights on at 2000 hours, off at 0800 hours). Food (Harlan Teklad, Madison, WI) and water were provided *ad libitum*, except during behavioral testing. All testing occurred between 1000 and 1800 hours; animal testing was conducted in accord with the 'Principles of Laboratory Animal

Care' NIH guidelines and were approved by the UCSD institutional animal care and use committee.

2. Drugs: Pramipexole (PRA) was purchased from Toronto Research Chemicals (North York, Ontario, Canada), and apomorphine hydrochloride hemihydrates (APO) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Drug doses are based on milligram/kilogram salts. All drugs were injected subcutaneously in a volume of 5 ml/kg body weight. Based on published studies in other mouse strains, PRA (saline vehicle, 0.01, 0.03, or 1.0 mg/kg) was administered 10 minutes before PPI testing (Maj et al., 1997; Siuciak and Fujiwara, 2004) in Experiment 1. Due to lack of any evidence of bioactivity of these doses of PRA in Exp. 1, Exp. 2 assessed whether the correct pretreatment time had been used by measuring effects of PRA (saline vehicle, 0.1, 0.3, 1.0 mg/kg) on PPI in the 60 minutes immediately after drug administration. In Exp. 3 and 4, doses were increased for PRA (Exp. 3: saline vehicle, 0.3, 1.0, 3.0 mg/kg; Exp. 4: saline vehicle, 10 mg/kg) and administered 15 minutes before PPI testing. APO (0.01% ascorbate/saline vehicle, 5 mg/kg) was administered 5 min before PPI testing (Exp. 4). A range of stimulus types was used in Exps. 1-4, described below, to attempt to identify PRA-sensitive parameters. In Exp. 5, PRA (saline vehicle, 0.3, 1.0, 3.0 mg/kg) was administered immediately before locomotion testing.

3. Startle apparatus: Startle reactivity was measured using four startle chambers (SR-LAB, San Diego Instruments, San Diego, CA). Each chamber consisted of a clear nonrestrictive Plexiglas cylinder (inner diameter = 3.8 cm) resting on a platform inside a ventilated box. A high-frequency loudspeaker inside the chamber produced both a continuous background noise of 65 dB(A) and the various acoustic stimuli. Vibrations of the Plexiglas cylinder caused by the whole-body startle response of the animal were transduced into analog signals by a piezoelectric unit attached to the platform. These signals were then digitized and stored by a computer. Sixty-five readings were taken at 1 ms intervals, starting at stimulus onset, and the average amplitude was used to determine the acoustic startle response (ASR). The SR-LAB calibration unit was used routinely to ensure consistent stabilimeter sensitivity between test chambers and over time, and sound levels in dB SPL (A scale) were measured as described previously (Geyer and Dulawa, 2003).

4. Startle testing procedure: All PPI test sessions consisted of startle trials (PULSE-ALONE), prepulse trials (PREPULSE + PULSE), and no-stimulus trials (NOSTIM). The PULSE-ALONE trial consisted of a 40 ms, 120 dB(A) pulse of broad-band noise. The NOSTIM trial consisted of background noise only. Parameters of PREPULSE + PULSE trials and the duration of the sessions varied with each Experiment. The test session began and ended with four to six presentations of the PULSE-ALONE trial; in between, each acoustic or NOSTIM trial type was presented in a pseudo-random order. There was an average inter-trial interval (ITI) of 15 s (range: 12-

30s). A background noise level of 65 dB(A) was presented for a 5-min acclimation period and continued throughout the matching and test sessions. 7 days after shipment arrival, mice were exposed to a 11 minute 'matching' startle session consisting of (10) PULSE-ALONE trails that were interspersed with (6) PREPULSE + PULSE trials consisting of PULSE-ALONE preceded 100 ms (onset-to-onset) by a 20 ms noise burst that was 81 dB(A) in intensity (i.e., 16 dB above the 65 dB(A) background). One "non-responder" was excluded from further testing due to a mean startle response to PULSE-ALONE trials of < 10 units during the matching session. Mice were assigned to drug treatment groups balanced for baseline PPI and startle magnitude during this matching session, as well as previous drug history. Groups were reassigned for each experiment. Test sessions were at least 1 week apart.

a. Exp. 1, 4: PREPULSE + PULSE trials consisted of PULSE-ALONE preceded 100 ms by a 20 ms noise burst that was 69, 73, or 81 dB(A) in intensity (i.e., 4, 8, or 16 dB above the 65 dB(A) background). Session duration was approximately 18.5 minutes.

b. Exp. 2: The test session was divided into 6 10-minute blocks after the 5 minute acclimation period. The initial 5 min of each block included four trial types: PULSE-ALONE, PREPULSE+PULSE (i.e. PULSE-ALONE preceded 100 ms by a 20 ms noise burst 8 dB above background), PREPULSE-ALONE, and NOSTIM.

c. Exp. 3: PREPULSE + PULSE trials consisted of PULSE-ALONE preceded 10, 20, 30, 60, 120 ms (onset-to-onset) by a 20 ms noise burst at 73 dB(A) (8 dB above background). Session duration was approximately 15.5 minutes.

5. Analysis of PPI Data: PPI was defined as 100-[(startle amplitude on PREPULSE trials/startle amplitude on PULSE-ALONE trials) $\times$ 100], and was analyzed by mixed design ANOVAs. All data were inspected for the presence of "non-responders" defined by a mean startle response to PULSE-ALONE trials of < 10 units; aside from one mouse excluded after the matching session, none met this criteria. Other ANOVAs were used to assess PULSE-ALONE magnitude, or NOSTIM trials. Post-hoc comparisons were conducted using Fisher's protected least significance difference, with the threshold for α set at 0.05. Data were collapsed across prepulse intensities and PPI blocks.

6. Locomotion apparatus: Investigatory behavior and locomotor activity were measured in 10 mouse Behavior Pattern Monitor (BPM) chambers (San Diego Instruments, San Diego, CA). The design of the mouse BPM system is based on the rat BPM (for a detailed description, see Geyer et al, 1986). The mouse BPM chamber is a clear Plexiglas box containing a 30×60 cm holeboard floor. Each chamber is enclosed in a ventilated outer box to protect it from light and ambient noise from outside the chambers. The chamber contains 11 1.4-cm holes (3 in the floor and 8 in the walls), each provided with an infrared photobeam to detect investigatory nose pokes (hole pokes). The

location of the mouse is obtained from a grid of 12×24 photobeams 1 cm above the floor. Rearing is detected by an array of 16 photobeams placed 2.5 cm above the floor and aligned with the long axis of the chamber. The status of photobeams is sampled every 55 ms. A change in the status of photobeams triggers the storage of the information in a binary data file together with the duration of the photobeam status. Subsequently, the raw data files are transformed into (x, y, t, event) ASCII data files comprised of the (x, y) location of the animal in the mouse BPM chamber with a resolution of 1.25 cm, the duration of each event (t), and whether a hole poke or rearing event occurred.

7. Locomotor/exploratory activity testing procedure: Exp. 5: Mice were tested in the dark and during the dark phase of their light cycle. The animals were brought into the testing room under black cloth 1 h before testing. During testing, a white noise generator produced background noise at 65 dB. Pretreatment and test injections were made under red lights in the testing room. Data were collected for 90 min. The chambers were cleaned thoroughly between testing sessions.

8. Analysis of locomotor activity: Horizontal locomotor activity was quantified as distance traveled. The number of hole pokes and rearings were calculated as measures of exploratory behavior. Data were examined using mixed-model ANOVAs with treatment as between-subject factors and time as a repeated measure. Specific *post hoc*

comparisons between the selected groups in each time block were done using Tukey's studentized range method. Statistical significance was assessed using an α-level of 0.05.

D. Results:

1. PPI: ANOVA of %PPI failed to reveal significant main effects of PRA dose in Exp. 1 (F=1.87, df 3, 35; NS) (Fig. 8.1). There were also no significant effects of PRA on startle magnitude (F=2.32, df 3, 35; NS) (Fig. 8.1, inset) or activity during NOSTIM trials (F=3.85, df 3, 35; NS), nor was there a significant block effect (F=13.96, df 3, 35; NS) (data not shown). As expected, there was a significant effect of prepulse intensity (F=116.07, df 6, 35; $p < 0.0001$), but no dose × intensity interaction (F < 1). The lack of effect of PRA on PPI was hypothesized to be due to one of three factors: 1) incorrect pretreatment time, missing the time period of maximal bioactivity, 2) incorrect prepulse to pulse time interval, or 3) inadequate dose of the drug. These three possibilities were addressed in Experiments 2-4. The total sample of mice was split between Experiments 2 and 3.

Figure 8.1: Effects of 0.1-1.0 mg/kg PRA on PPI and startle magnitude (inset) across varying prepulse intensities in C57BL/6J mice (Exp. 1). Startle magnitude and prepulse inhibition with prepulses 4, 8, and 16 dB(A) above background were tested 10 minutes after administration of 0.1, 0.3, and 1.0 mg/kg of PRA. ANOVA of PPI revealed significant effects of prepulse intensity ($p < 0.0001$), but no significant effect of drug dose and no dose × intensity interaction. PRA also had no significant effect on startle magnitude. (n = 9-10 mice per dose group)

In Experiment 2, PPI was measured in six 10-minute blocks, starting 5 minutes after administration of PRA. Again, there were no significant main effects of PRA ($F < 1$) (Fig. 8.2), and there was no dose × block interaction ($F < 1$). As in Exp. 1, there was no significant effect of PRA dose on startle magnitude ($F=2.31$, df 3,16; NS) (Fig. 8.2, inset).

Figure 8.2: Effects of 0.1-1.0 mg/kg PRA on PPI and startle magnitude (inset) across 60 minutes of testing in C57BL/6J mice (Exp. 2). To assess the time course for maximal bioactivity, PPI was tested in 6 10-minute time blocks after administration of 0.1, 0.3, and 1.0 mg/kg PRA. ANOVA of PPI failed to reveal a significant effect of PRA dose, and no dose × time block interaction. Again, PRA had no significant effect on startle magnitude. As in Exp. 1, there was no evidence of bioactivity of PRA at these doses. (*n = 5* mice per dose group)

In Experiment 3, the interval between the onset of the (constant intensity) prepulse and the pulse was varied between 10, 20, 30, 60, and 120 msec. PRA doses were also increased to 0.3, 1.0, and 3.0 mg/kg. As expected, there was a significant effect of prepulse interval (F=11.67, df 4, 15; p <0.0001) in the pattern that has been reported in

rats (e.g. Swerdlow et al., 2009a). Again, ANOVA failed to reveal a significant main effect of PRA on PPI (F <1), significant dose × interval interaction (F=1.37, df 12, 15; NS) (Fig. 8.3) or significant effect of PRA on startle magnitude (F = 1.95, df 3, 15; NS) (Fig. 8.3, inset).
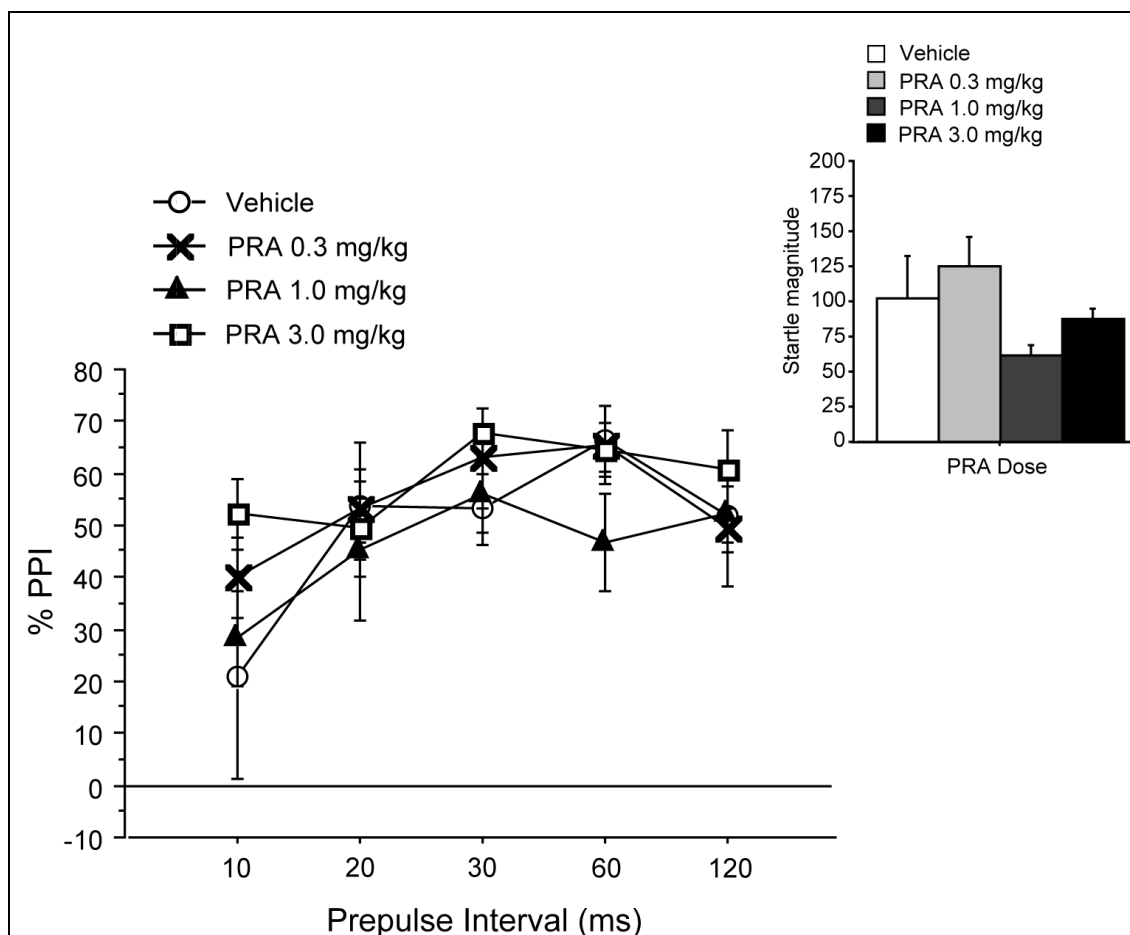
Figure 8.3: Effects of 0.3-3.0 mg/kg PRA on PPI and startle magnitude (inset) across varying prepulse time intervals in C57BL/6J mice (Exp. 3). Due to the absence of evidence of bioactivity in Exps. 1 and 2, PRA doses were shifted higher to 0.3, 1.0, and 3.0 mg/kg for Exp. 3. Prepulses were 8 dB(A) above background, and preceded the pulse by 10, 20, 30, 60, or 120 ms (onset-to-onset). ANOVA of PPI revealed a significant main effect of prepulse interval ($p < 0.0001$), but no effect of PRA dose, and no significant dose × interval interaction. Even at the highest dose, there was no significant effect on startle magnitude. (n = 4-5 mice per dose group)

For the final PPI experiment (Exp. 4), the two groups from Exps. 2 and 3 were recombined. Mice were regrouped to balance for baseline PPI and drug history. As no evidence of bioactivity had yet been demonstrated for any doses of PRA used in Exps. 1-3, the PRA dose was increased to 10 mg/kg. Apomorphine (APO, 5 mg/kg) was used as a reference drug to determine whether testing was adequately sensitive to detect a dopaminergic regulation of PPI. ANOVAs revealed a significant main effect of drug on %PPI ($p < 0.0001$). Post hoc tests revealed a significant reduction in %PPI after APO ($p < 0.0001$), but not after 10 mg/kg PRA (NS) (Fig. 8.4). However, there was also a significant main effect of drug on startle magnitude ($p < 0.0001$), and post hoc tests indicated that both PRA ($p < 0.009$) and APO ($p < 0.04$) significantly reduced startle magnitude (Fig. 8.4, inset). Additionally, it was observed grossly that mice receiving 10 mg/kg of PRA appeared lethargic and had reduced activity as soon as 5 minutes after drug administration.

Figure 8.4: Effects of 10 mg/kg PRA and 5 mg/kg APO on PPI and startle magnitude (inset) across varying prepulse intensities in C57BL/6J mice (Exp. 4). PRA was administered at a maximum dose of 10 mg/kg for comparison against 5 mg/kg of APO with the same test parameters used in Exp.1. ANOVA of PPI revealed the expected main effect of intensity ($p < 0.0001$), and a significant main effect of drug ($p < 0.0001$). Fisher's PLSD indicated that PPI scores of mice treated with vehicle were significantly different from APO- but not PRA-treated mice. There was no drug $\times$ intensity interaction. Both 10 mg/kg PRA and 5 mg/kg APO significantly reduced startle magnitude to PULSE-ALONE trials. Thus, in Exp. 5, 10 mg/kg PRA demonstrated evidence of bioactivity via startle reduction with no change in PPI, and APO served as a positive control of dopamine agonist-induced PPI disruption. *$p < 0.0001$, #$p < 0.009$, ^$p < 0.04$. ($n = 9$ mice per dose group)

2. Locomotor and exploratory activity: In Exp. 5, a different set of mice were tested for locomotor activity at doses of PRA (0.3, 1, 3 mg/kg) that did not demonstrate any effects on either PPI or startle magnitude. ANOVAs revealed a significant main effect of drug dose ($p < 0.0001$) and dose × time interaction ($p < 0.0001$) on locomotor activity and both measures of exploratory activity. All doses caused significant reductions in locomotor activity during the first 30 minutes after administration compared to the vehicle condition. At 0.3 mg/kg PRA, mice showed decreased locomotor activity during the first 30 minutes and hyperactivity during the last 40 minutes compared to the vehicle condition. Mice receiving 1.0 mg/kg PRA had decreased locomotor activity during the first 40 minutes that gradually increased to similar levels as vehicle-treated mice for the remainder of the 90-minute testing period. At the highest dose of PRA tested, 3.0 mg/kg, mice showed a steady and decreased level of locomotor activity throughout the testing period. Habituation -- reduced locomotor activity in vehicle-treated mice as they acclimated to the BPM chamber -- was not observed in mice treated with PRA (Fig. 8.5a).

ANOVA of hole pokes revealed a significant main effect of drug dose, with a steady, dose-dependent decrease in the number of hole pokes throughout the 90 minute test session (Fig. 8.5b). There was no significant dose × time interaction ($F = 1.164$, df 24, 32; NS). The pattern of rearing looked similar to that of locomotion, with a significant dose × time interaction ($p < 0.0001$). The group treated with 0.3 mg/kg PRA demonstrated fewer rears during the first 40 minutes, which increased by the end of the test session. Mice treated with 1.0 or 3.0 mg/kg PRA reared less than saline treated mice

for the entire session, though this difference failed to reach significance for the last time

block of the middle dose, due to a slight increase in rears by this treatment group as well

as a gradual decrease in rears by vehicle-treated mice. (Fig. 8.5c).
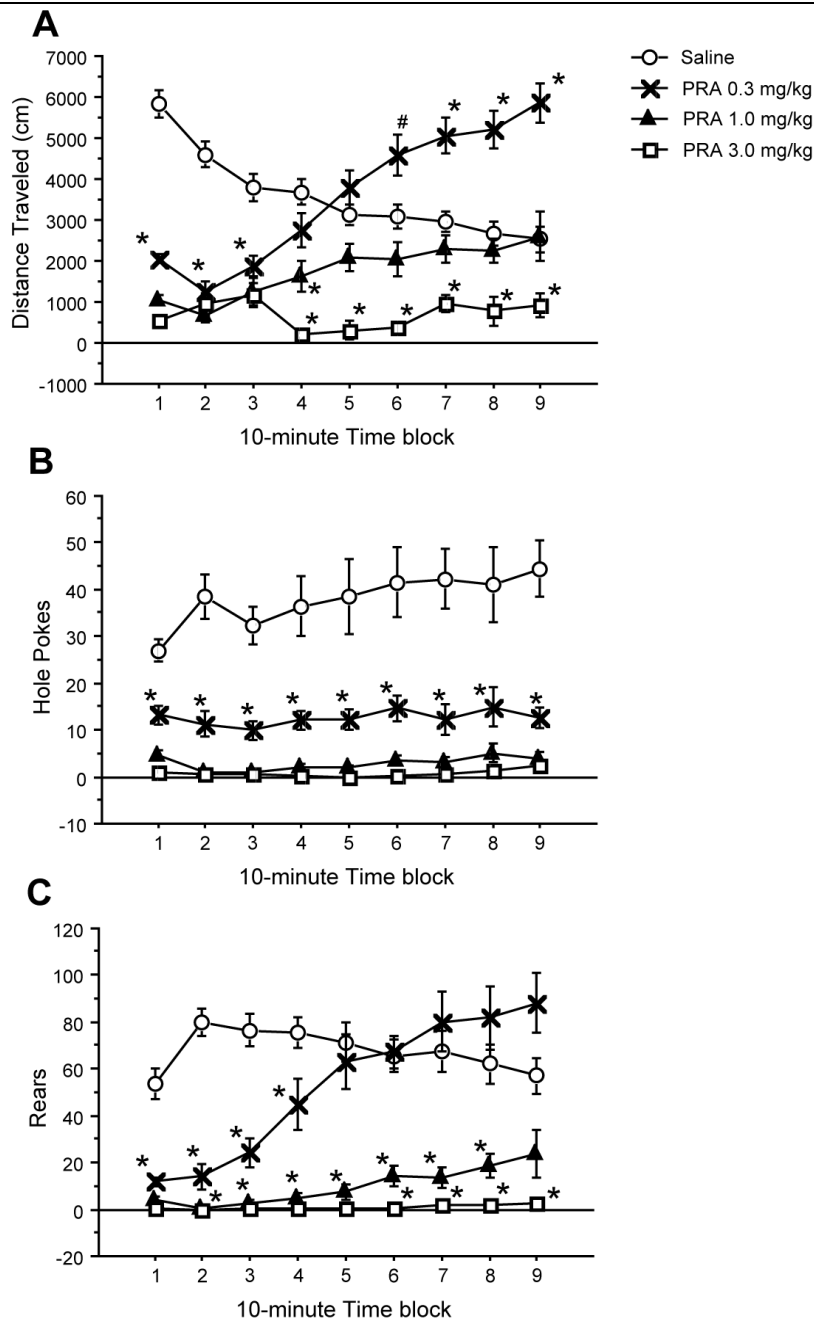
Figure 8.5: Effects of PRA on locomotor activity (A) and exploratory activity (B,C). Nonacclimated, drug-naïve mice were placed in Behavior Pattern Monitor (BPM) chambers immediately after administration of saline vehicle or 0.3, 1.0, or 3.0 mg/kg of PRA. Locomotor and exploratory behavior was measured for 90 minutes, divided into 10 minute time blocks. (a) Dose response of PRA effects on distance traveled (in cm). Mice used were male C57BL/6J. (b) Dose response of PRA effects on number of hole pokes. (c) Dose response of PRA effects on number of rears. Data are mean ± SEM. $*p < 0.01$, $\#p < 0.05$. ($n = 9$ mice per dose group)

E. Discussion:

The present studies expand upon previous reports of DA agonist effects on PPI and locomotion in mice by testing the preferential D3 agonist pramipexole for behavioral effects in C57BL/6J mice. The ability of APO to reduce PPI confirms several previous findings in this mouse strain (Caldwell et al., 2009; Martin et al., 2008; Ralph-Williams et al., 2002, 2003; Russig et al., 2005, Semenova et al., 2008; van den Buuse et al., 2005; Yee et al., 2004) and other strains (Brea et al., 2006; Curzon and Decker, 1998; Depoortère et al., 2007; Malone et al., 2004; Park et al., 2005; Ukai and Okuda, 2003; Yano et al., 2009). While no effect of PRA on PPI was shown in this report, bioactivity was demonstrated by a reduction in startle magnitude at the highest dose tested (10 mg/kg) and by changes in locomotor and exploratory behavior across lower doses. Startle magnitude reduction after administration of PRA at doses inducing PPI deficits is often observed in rats (Weber et al., 2008a, 2009b). A reduction in startle magnitude (indicating bioactivity) in the absence of any PPI effects in these C57BL/6J mice suggests that the pharmacological actions of PRA do not regulate PPI in this strain/species. Indeed, the mixed D2/D3 agonists quinpirole and quinelorane fail to disrupt PPI in several strains of mice, including C57BL/6J (Ralph and Caine, 2005; Ralph-Williams et al., 2003). It should be noted, though, that quinelorane can disrupt PPI in some mouse strains (Ralph and Caine, 2007), and quinpirole has been shown to increase PPI in mice when infused directly into the nucleus accumbens. In contrast, rats reliably exhibit reduced PPI after systemic pramipexole, quinpirole, or quinelorane administration, at least at the most commonly used prepulse intervals (Culm et al., 2004;

Qu et al., 2008; Ralph and Caine 2005, Weber et al., 2008a). It has been reported through knockout studies in mice that the DA D2 receptor (D2R) subtype is necessary for amphetamine-induced PPI disruptions, while the DA D1 (D1R), D3 (D3R), and D4 (D4R) receptor subtypes are not (Ralph et al, 1999). D3R knockout mice have been shown to have exaggerated effects of cocaine on PPI (Doherty et al., 2008) perhaps due to unopposed effects of D1R activation. However, preferential D3R agonists and antagonists do not always produce effects that are consistent with specific genetic manipulations of receptor subtypes (Corbin et al., 1998; Le Foll et al., 2002; Xi et al, 2005). These results suggest that, while PRA may primarily be stimulating D3Rs in mice, this pharmacological activation does not alter PPI, and furthermore, D3Rs may not play a prominent role in PPI regulation in this mouse strain.

Locomotion and exploratory behavior findings in the present studies are in keeping with previously published reports on pramipexole effects in other strains of mice (Lehr et al., 2002; Maj et al., 1997; Siuciak and Fujiwara 2004). 7-OH-DPAT and PD 128907, two other D3-preferential DA agonists, also reduce locomotor activity in C57BL/6 mice; this effect is not present in D3 receptor knockout mice (Pritchard et al., 2003). Additionally, D3 receptor antagonists enhance locomotor hyperactivity due to cocaine (Piercey et al., 1992) and amphetamine (Pritchard et al., 2007), and mice deficient in D3 receptors exhibit an augmented cocaine-stimulated locomotion. Thus, compared to their role in regulating PPI in mice, D3 receptors appear to play a more prominent role in the regulation of locomotor activity.

The main goal of this study was to establish an assay in mice that could be used via molecular manipulations to assess the D3 specificity of PRA in its impact on PPI. Our findings, however, suggest that such an assay may not be informative, based on the surprising insensitivity of PPI to apparent D3 stimulation in mice. While "negative results" must always be interpreted with caution, this study used a number of different approaches to demonstrate that the lack of PRA effects on PPI did not reflect the insensitivity of the PPI paradigm, lack of PRA bioactivity, or a restricted range of stimulus parameters or timecourse. In so doing, it is conceivable that these results identify a fundamental difference in the dopaminergic regulation of PPI across rat vs. mouse species, analogous to other species differences in PPI previously reported in rats vs. mice (Ralph and Caine, 2005; Ralph-Williams et al., 2002, 2003). If molecular manipulations are to be applied towards clarifying the basis for the PPI-disruptive effects of D3 stimulation, it would appear that such studies may need to be pursued in rats, or at least in a different mouse strain.

F. Acknowledgements:

CHAPTER 9:

The effects of pramipexole on locomotor activity in D3 mutant mice

Wei-li Chang, Neil M. Richtand, Rebecca Ahlbrand, Neal R. Swerdlow

A. Abstract:

We previously reported that pramipexole (PPX), a preferential dopamine D3 receptor (D3R) agonist, has dose- and time-dependent effects on locomotor and exploratory behavior in C57BL/6J mice. Here, we confirmed these locomotor effects of PPX and tested whether they are dependent on D3R stimulation in D3R knockout (D3KO) and wild-type (WT) mice. The contribution of D2Rs to PPX effects was also examined using the D2-selective antagonist L741626. In all mice, higher doses of PPX (1.0 – 3.0 mg/kg) elicited sustained hypolocomotion, while the lowest dose (0.3 mg/kg) produced a biphasic effect: early hypolocomotion and late hyperlocomotion. Hyperlocomotion was more pronounced and occurred sooner in D3KO vs. WT mice. D2 blockade with L741626 opposed PPX-induced hyperlocomotion in WT and D3KO mice. This pharmacologic, genetic and behavioral parsing suggests a subtle but consistent functional opposition of D3R and D2R systems in the regulation of PPX-induced locomotor behavior: specifically, D3R activation appeared to oppose a late-emerging D2R-dependent hyperlocomotion.

B. Introduction:

The dopamine D3 receptor has been implicated as a potential contributor to the pathophysiology of several neuropsychiatric disorders, and as a novel target for pharmacotherapeutics, but such assertions are weakened by difficulties in validating the D3-specificity of currently available pharmacological tools. For example, the structures of D3 and D2 receptors have 52% homology in amino acid sequences, with 75% homology in transmembrane domains (Sokoloff et al., 1990), yet studies attempting to identify D3-mediated behavioral or pharmacological signals must convincingly dissociate D3 signals from those attributable to D2 receptors; the latter are very prevalent throughout the forebrain, and are targets of all clinically-effective antipsychotics. Only a small number of well-characterized drugs are thought to preferentially act at D3 vs. D2 receptors, but evidence for even such receptor preference comes from experimental measures based on overlapping gradients of D3 vs. D2 dependence, rather than clear, categorically dissociable markers.

Pramipexole (PPX) is a dopamine D2/D3 receptor full agonist that has a D3:D2 binding preference ranging from 7:1 to 160:1 *in vitro* (Millan et al., 2002; Piercey et al., 1996; Svensson et al., 1994a). In humans, PPX enhances specific neurocognitive functions among individuals with low basal levels of peripheral DRD3 expression, an effect attributed to an inverted-U relationship between levels of forebrain D3 activation and cognitive function (Ersche et al., 2011). While pharmacological tools have been employed to parse D3 vs. D2 receptor effects of PPX in rodents, only a small number of studies have applied *in vivo* molecular manipulations to this goal. Studies using D3

knockout mice suggested a role for D3 receptor activity in the PPX-induced downregulation of the dopamine transporter, which is neuroprotective against MPTP toxicity (Joyce et al., 2004). However, studies of D3 knockout mice with both C57BL/6J and B6129SF2/J background strains suggested that PPX-induced reductions in immobility during the forced swim test were due to D2 rather than D3 effects (Siuciak and Fujiwara, 2004).

We previously reported that PPX alters locomotor and exploratory behavior in C57BL/6J mice (Chang et al., 2010a), producing a biphasic pattern: an early suppression of locomotion, followed by a dose-dependent, long-latency hyperactivity. However, the contribution of D3 vs. D2 receptor activation to these effects was not established. Two other D3-selective agonists, 7-OH-DPAT and PD 128907, are also known to decrease locomotor activity in a novel environment in C57BL/6J mice (Xu et al., 1999; Boulay et al., 1999b; Pritchard et al., 2003). 7-OH-DPAT has an *in vitro* D3:D2 binding preference ranging from 5:1 to 178:1 (Sautel et al., 1995; Levant, 1997), and PD 128907 has a binding preference ranging from 18:1 to 53:1 (Pugsley et al., 1995, Sautel et al., 1995). As with PPX, the dependence on D3 vs. D2 receptor activity in the observed behavioral effects of 7-OH-DPAT and PD 128907 *in vivo* has been difficult to discern (Daly and Waddington, 1993; Ahlenius and Salmi, 1994; Pugsley et al, 1995; Levant et al, 1996; Bancroft et al, 1998; Xu et al, 1999; Boulay et al, 1999a, 1999b). For example, 7-OH-DPAT and PD 128907 produce locomotor-suppressive effects in both WT and D3 KO mice (Xu et al., 1999; Boulay et al., 1999b), though perhaps at doses that lack specificity for D3 vs. D2 receptors. At lower doses thought to minimize D2 receptor occupancy,

Pritchard et al. (2003) demonstrated a loss of the locomotor-suppressive effects of 7-OH-DPAT and PD 128907 in D3 KO mice.

In saline-treated mice, a high level of locomotor activity immediately after placement into the novel testing chamber is followed be a gradual decrease in activity as they become acclimated to the chamber. In previous studies (Chang et al., 2010a), higher doses of PPX (1.0 – 3.0 mg/kg) decreased locomotor and exploratory behavior in mice across 90 min of testing. Interestingly, a lower dose of 0.3 mg/kg PPX produced hypolocomotion for 30 min after drug injection, followed by a gradual increase in activity that resulted in hyperlocomotion, 50-90 min after drug injection. This biphasic low-dose effect may suggest that opposing processes are initiated by multi-receptor effects of PPX, and that the mechanisms driving the low-dose hyperactivity can be functionally overwhelmed by opposing mechanisms at higher PPX doses. To determine the relative contribution of D3 vs. D2 receptor activation to PPX-induced hypo- vs. hyperactivity, we measured exploratory locomotor activity after PPX in C57BL/6J-derived mice lacking functional D3 receptors. Because the present studies were conducted in a different city (Cincinnati vs. San Diego) using a different (smaller) locomotor monitoring apparatus, initial studies were conducted to confirm comparable PPX effects to those reported previously (Chang et al., 2010a).

C. Methods:

1. Mice: For initial studies testing PPX effects in mice as in Chang et al. (2010a), male and female C57BL/6J mice (n=67) 6 weeks of age were obtained from Jackson Labs (Bar Harbor, Maine, USA) and housed at a vivarium at the Cincinnati Department of Veterans Affairs Medical Center. Mice were allowed to acclimate for approximately 2 weeks after arrival.

For studies involving D3 receptor knockouts, mice heterozygous for the D3 receptor mutations were bred to generate the mice used in this study, and were offspring of mice used in previous D3 agonist studies (Pritchard et al., 2003; Xu et al., 1997). Genetic backgrounds of both mutant and WT mice were C57BL/6 × 129Sv, and had been subsequently bred for seven generations with C57BL/6 mice. At weaning, males and females were separated and littermates housed together, unless there were less than 3 littermates from either sex, in which case they were combined with mice from another litter. Genotypes were confirmed by polymerase chain reaction (PCR).

For all studies, there were no more than 5 animals per cage, under controlled temperature and humidity on a 12-h light/dark cycle (0600 on, 1800 off for Jackson mice; 2400 on, 1200 off for KO/WT mice). Food and water were available *ad libitum*. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, and were approved by the Cincinnati Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

2. Drugs: Pramipexole (PPX) was purchased from Toronto Research Chemicals (North York, Ontario, Canada), and L741626 from Tocris (Ellisville, MO, USA). Drug doses are based on mg/kg of solid and administered subcutaneously in a volume of 5 ml/kg. PPX (0, 0.3, 1.0, 3.0 mg/kg) was dissolved in saline, and L741626 (vehicle, or 0.6 mg/kg) was dissolved in 0.15% lactic acid/water (w/v) and pH was adjusted to ≥5 using NaOH. PPX was administered immediately before placement into testing chambers. For studies using a L741626 pretreatment, drug (vehicle or 0.6 mg/kg) was administered 30 min before PPX injection (vehicle, 0.3 or 1.0 mg/kg) to WT and mutant mice previously tested with PPX (0 – 3.0 mg/kg) alone, using dose groups randomized for previous PPX exposure. The dose of 0.6 mg/kg L741626 was selected based on other reports (Millan et al., 2000a, 2004a; Brocco et al., 2006), where it was shown to block several behaviors linked to D2-receptor activation while having a minimal effect on unhabituated locomotor activity. At higher doses, L741626 has been found to independently suppress locomotor activity (Millan et al., 2000a).

3. Apparatus: Behavioral testing was performed in 30 custom-designed residential activity chambers (RACs), modeled after chambers designed by Segal and Kuczenski (1987), and as described in Pritchard et al., 2003. These chambers were configured slightly differently from those used in our previous studies of PPX-locomotion in mice (Chang et al., 2010a): each chamber consists of a ventilated, sound-attenuated cabinet (Cline Builders, Covington, KY) containing a $40 \times 40 \times 38$ cm^3 Plexiglas enclosure. A fan in each enclosure provides air circulation and constant

background noise. All behavioral testing was performed during the "lights-off" portion of the light cycle (between 1200 and 2400). Locomotion was monitored with a 16×16 photo beam array (San Diego Instruments, San Diego, CA) located 1.25 cm above the floor of the enclosure. Locomotion is expressed as crossovers, defined as entry into any of the "active zones" of the chamber, as shown in Fig. 9.1. Distance travelled and beam break counts were also assessed.

Figure 9.1: Schematic of the Residential Activity Chamber (RAC). Dotted lines represent the 16×16 photo beam array used to record location of the mouse. Shaded areas in the figure correspond to "active zones" used for tabulating crossovers. The RAC floor has no color variations; shading here is solely for illustrative purposes.
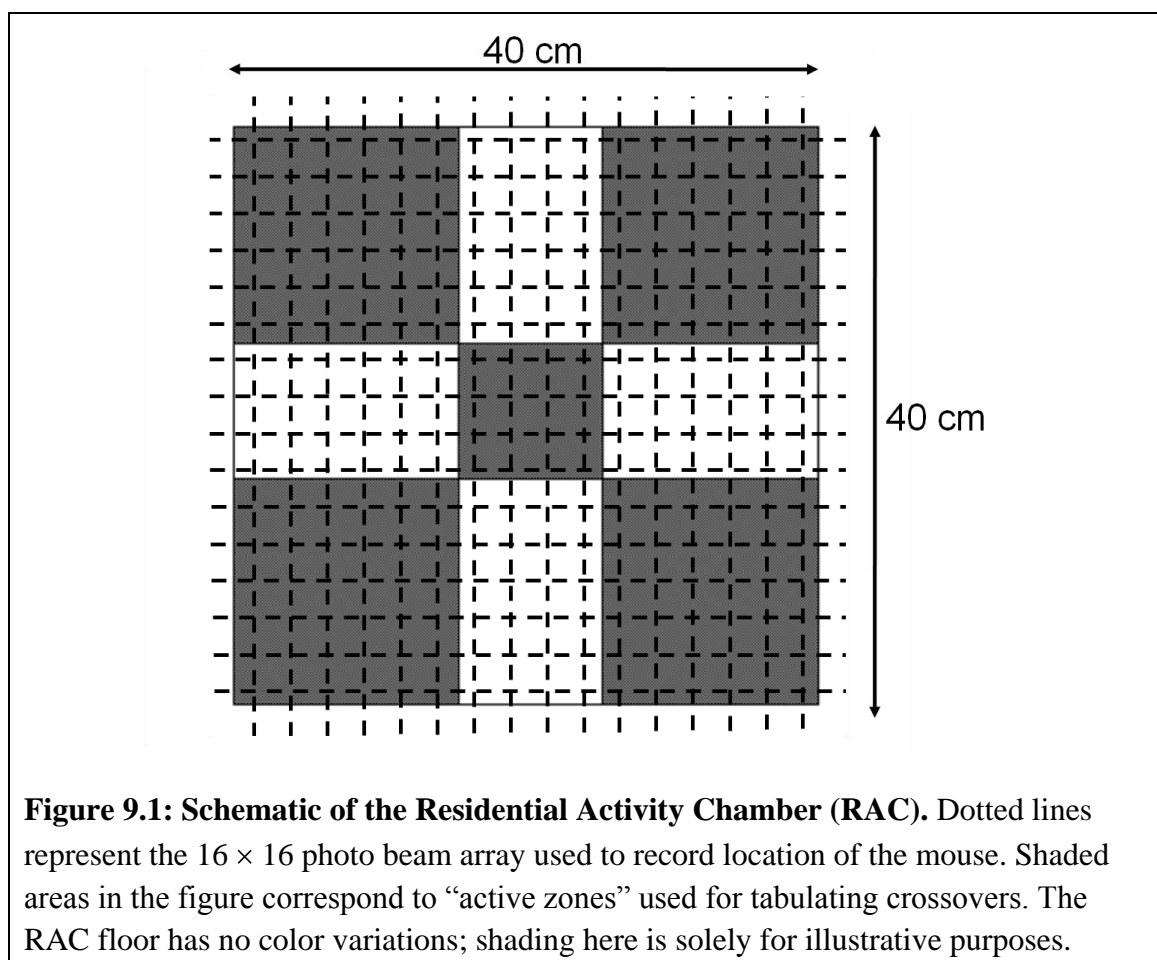
4. Behavioral Testing Procedure: Male and female mice, 6-9 weeks old, were tested on separate days. On the first day of testing, mice without prior RAC exposure were removed from home cages and injected with saline or PPX (0.3, 1.0, 3.0 mg/kg, sc) and immediately placed in the RAC. Mice were re-tested after 1 week with a pretreatment injection of water/0.15% Lactic Acid vehicle or L741626 (0.6 mg/kg, sc) and placed into individual cages for 30 minutes before injection with PPX (0, 0.3 mg/kg) and placement into the RAC. Locomotion was then tested for 90 minutes after injection.

5. Statistical Analysis: Locomotor activity was collected in 10 min bins for 90 minutes. Results are expressed as group mean ± SEM of total crossovers. Other measures (e.g. beam breaks, distance travelled) yielded comparable results but are not reported here to avoid redundancy. After analyses to characterize the independent effects of PPX and D3 genotype on locomotor activity across the full 90 min time-course, our *a priori* design was to conduct analyses of PPX, genotype and their interactions during the initial and final 30 minute blocks of the 90 minute test; this design was based on our previous findings of a biphasic temporal pattern with the lowest dose of PPX (0.3 mg/kg), which suggested that distinct mechanisms might regulate early and late effects of PPX (Chang et al., 2010a). Within-genotype comparisons were made by repeated-measures ANOVAs, and post-hoc comparisons were conducted using Fisher's PLSD. Alpha was < 0.05.

D. Results:

1. Effects of pramipexole on locomotor activity in WT C57BL/6 mice: Findings confirmed a biphasic hypo- and then hyper-locomotion effect of 0.3 mg/kg PPX and sustained hypolocomotion in animals treated with 1.0 or 3.0 mg/kg; compared to our previous findings in larger activity monitors, the onset of hyperlocomotion was slightly delayed (starting at 80 min instead of at 50 min) and less pronounced (Fig. 9.2; Chang et al., 2010a). Repeated-measures ANOVA of crossovers revealed significant main effects of PPX dose (F=62.52, *df* 3,59; *p*<0.0001) and time interval (F=15.67, *df* 8, 472; *p*<0.0001), and a significant PPX × interval interaction (F=24.05, *df* 24,472; *p*<0.0001). There was no significant effect of sex, and no interactions between sex and PPX dose, time interval, or dose × interval. Data were collapsed across sexes for further analyses. Post-hoc tests indicated that all doses of PPX significantly reduced locomotor activity for the first 60 min of testing, which continued in the 1.0 and 3.0 mg/kg dose groups for the remainder of the session. Over the final test intervals, animals treated with 0.3 mg/kg PPX demonstrated a significant increase in locomotor activity, and during the last 10 min of testing, these levels were significantly greater than those in vehicle-treated mice (p<0.05).

Figure 9.2: Effects of pramipexole (PPX) on locomotor activity. Nonacclimated, drug-naïve mice were placed in Residential Activity Chambers (RACs) immediately after administration of saline vehicle of 0.3, 1.0, or 3.0 mg/kg of PPX. Locomotor activity was measured for 90 min, divided into 10-min time blocks, replicating a previously reported study conducted in Behavior Pattern Monitor chambers (Chang et al., 2010a). Mice were adult male and female C57BL/6J. Data are mean ± SEM. *$p<0.001$, +$p<0.05$.

2. Effects of DRD3 mutation on basal locomotor activity: Locomotor levels in saline-treated male and female WT and KO mice were first tested for an effect of genotype (Fig. 9.3). Repeated measures ANOVA showed no significant effects of genotype (F>1) or sex (F>1) and no significant interactions involving genotype. There was a significant effect of interval (F=66.18, *df* 8, 160; $p<0.0001$) and an interval × sex interaction (F=2.19, *df* 8,160; $p<0.04$) reflecting a more pronounced decrease in locomotor activity in females during the second half of the test session, but no significant sex differences at any single time interval was detected by post-hoc analyses.

Figure 9.3: Basal Locomotor Activity. There were no significant differences in locomotor activity during 90 min of observation between D3 KO and WT mice receiving saline injection. Data here are collapsed across time intervals. (n = 12 for D3 KO and WT mice)
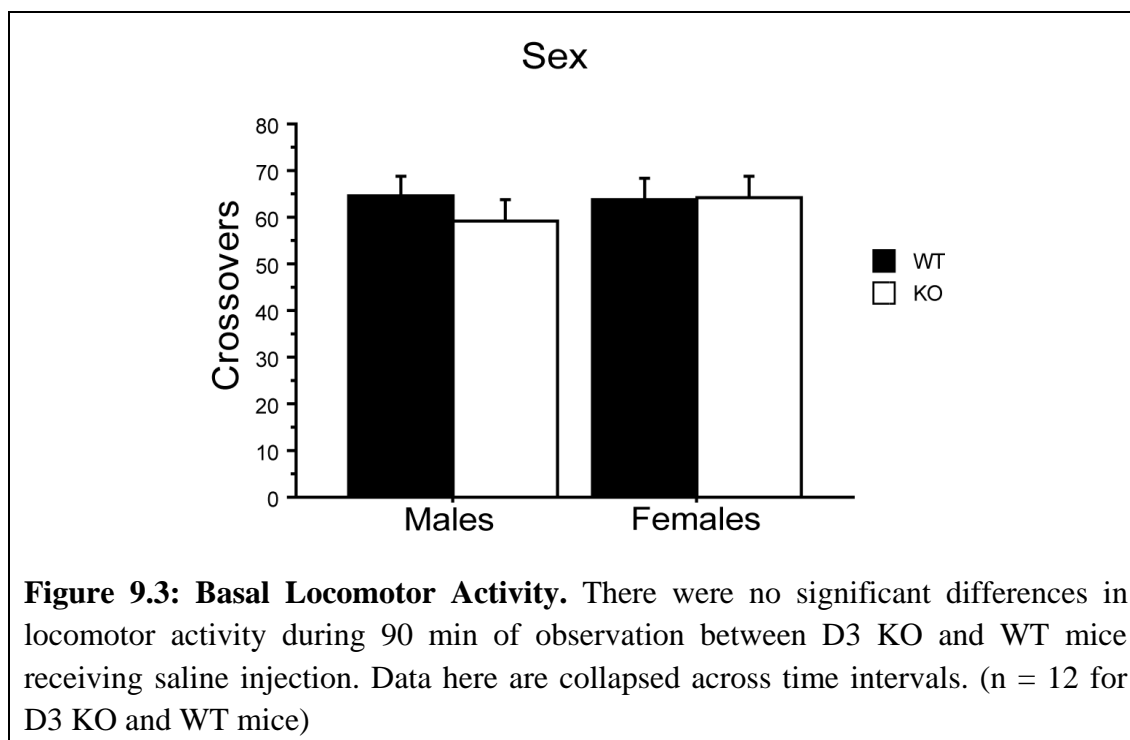
3. PPX effects on locomotor activity in WT and DRD3 mutant mice: In WT mice, ANOVA of crossovers revealed significant main effects of PPX (F=13.63, *df* 2,26; *p*<0.0002) and interval (F=10.02, *df* 8,208; *p*<0.0001). There was no effect of sex (F<1). There was a significant PPX × interval effect (F=13.34, *df* 16,208; *p*<0.0001), but no other significant interactions. Data were then collapsed across sexes for post-hoc analyses, which revealed a significant reduction in locomotion in the PPX 0.3 mg/kg group in the first 40 min (*p*<0.05), that waned but did not produce significant hyperlocomotion at any interval in the test session (Fig 9.4, left panel).

Compared to the WT mice, the PPX response in DRD3 mutant mice was characterized by a blunted and shorter-lived early hypoactivity, which was replaced after 40 min by a significant hyperlocomotor response. ANOVA of crossovers in DRD3

mutant mice revealed significant main effects of PPX (F=7.00, *df* 2,27; *p*<0.004) and interval (F=3.91, *df* 8,216; *p*<0.0003). In contrast to the WT mice, in mutant mice there was a significant effect of sex (female > male; F=7.62, *df* 2,27, *p*<0.02), but there were no significant interactions of sex × PPX, interval, or PPX × interval, so data were collapsed across sexes for further analysis. Fisher's PLSD, split by time interval, demonstrated a significant reduction of locomotion in the PPX 0.3 mg/kg group during first 20 min (*p*<0.05), and significant hyperlocomotion in the last 20 min of testing (*p*<0.05, Fig. 9.4, right panel).

Based on our *a priori* (Chang et al., 2010a) hypothesis of distinct early vs. late behavioral effects of this low dose of PPX (also confirmed in the present animals), we next directly compared the impact of D3 genotype on the locomotor effects of 0.3 mg/kg PPX during the initial and final 30 min of this 90 minute test session. ANOVA of activity during session minutes 0 - 30 revealed significant locomotor-*reducing* effects of PPX (F=48.74, *df* 1,37; *p*<0.0001), no significant effect of genotype (F<1), and no significant interaction of dose × genotype (F<1). Thus, D3 genotype did not appear to have a major impact on the initial, hypolocomotor phase of this biphasic drug effect. ANOVA of activity during session minutes 60 - 90 revealed significant locomotor-*potentiating* effects of PPX (F=7.35, df 1,37, *p*<0.015), and near-significant main and interaction effects of genotype (F=3.22, df 1,37, *p*<0.10) and dose × genotype (F = 2.88, df 1,37, *p*<0.10), respectively. While the dose × genotype interaction reached trend levels only, post-hoc comparisons revealed significant PPX-induced hyperlocomotion in D3 mutant (*p*<0.03) but not WT mice (*p*>0.45; NS). Thus, D3 genotype appeared to impact the late

PPX hyperlocomotor phase, in that mutation of the D3 gene enhanced - or perhaps "released" - PPX-induced hyperlocomotion.
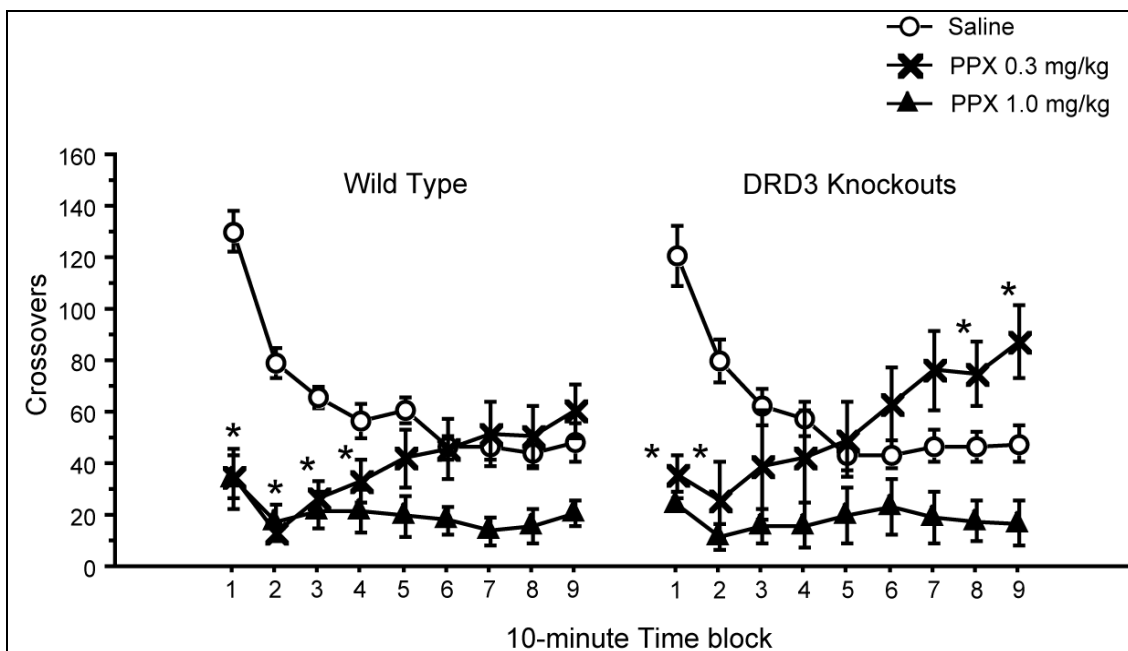
Figure 9.4: PPX effects on locomotor activity in D3 KO and WT mice. Overall, changes in locomotor activity after PPX administration are observed in both D3 KO and WT mice. In the lower dose tested, 0.3 mg/kg, early hypolocomotion is shortened by 20 minutes in the D3 KOs. Additionally, there is a significant hyperlocomotion effect observed during the last 20 minutes of the D3 KO but not the WT group. Data are collapsed across males and females. *$p<0.05$.

4. L741626 vs. PPX effects on locomotor activity: The above results with a *genetic* manipulation suggest that D3 mechanisms may not regulate the early hypolocomotor effects of PPX in mice, but appear to oppose (or at least modulate) a later PPX-induced hyperlocomotor response. A *pharmacologic* manipulation was next used to assess a potential D2R contribution to the motoric effects of PPX: the D2-selective

antagonist L741626 was administered prior to PPX injection. In WT mice, L741626 had no significant effect on the previously observed locomotor response to 0.3 mg/kg PPX. ANOVA revealed no significant effects of sex, L741626, or PPX on locomotor activity. There was a significant main effect of time interval (F=13.38, *df* 8,120; *p*<0.0001) and a PPX × interval interaction (F=23.28, *df* 8,120; *p*<0.0001), but no interactions of L741626 × PPX or L741626 × PPX × interval. Interestingly, on this second day of testing, WT mice pretreated with L741626 vehicle exhibited a PPX response more consistent with what was previously exhibited by the mutant mice: a relatively abbreviated early hypolocomotion followed by a later hyperlocomotion. In post-hoc analyses, the hypolocomotion effect was significant in the first 20 minutes of testing, and the hyperlocomotion effect was significant for the last 30 min; PPX-induced early hypolocomotion was statistically significant in both L741626 vehicle- and active-dose groups (effect of PPX: F>65, *p*<0.0001; effect of L741626 and PPX × L741626 interactions both F's<1), while the later PPX-induced hyperlocomotion was statistically significant only among mice pretreated with vehicle (p<0.025). Inspection of the data revealed an attenuation of the PPX hyperlocomotion effect in mice pretreated with 0.6 mg/kg vs. vehicle L741626 (Fig. 9.5), though this did not reach statistical significance.

Among D3 KO mice, there were also no significant effects of sex, L741626, or PPX on locomotor activity. As in the WT mice, there was a significant main effect of interval (F=14.35, *df* 8,112; *p*<0.0001) and a significant PPX × interval interaction (F=25.79, *df* 8,112; *p*<0.0001), but no interactions of L741626 × PPX or L741626 × PPX × interval. Among KO mice pretreated with vehicle, PPX significantly reduced

locomotor activity for the first 20 min of testing, and the later PPX-induced hyperlocomotion was pronounced and statistically significant for the last 50 min of testing. Thus, similar to what was observed on the first day of testing, compared to WT mice, D3KO mice exhibited an earlier and more robust emergence of PPX-induced hyperlocomotion. Among D3 KO mice, this later PPX-induced hyperlocomotion was significantly opposed by L741626 (Fig. 9.5): during the final 60 minutes of testing, among PPX-treated mice, ANOVA revealed a significant effect of L741626 pretreatment (F=5.24, *df* 1,9; *p*<0.05), but no L741626 $\times$ interval interaction.
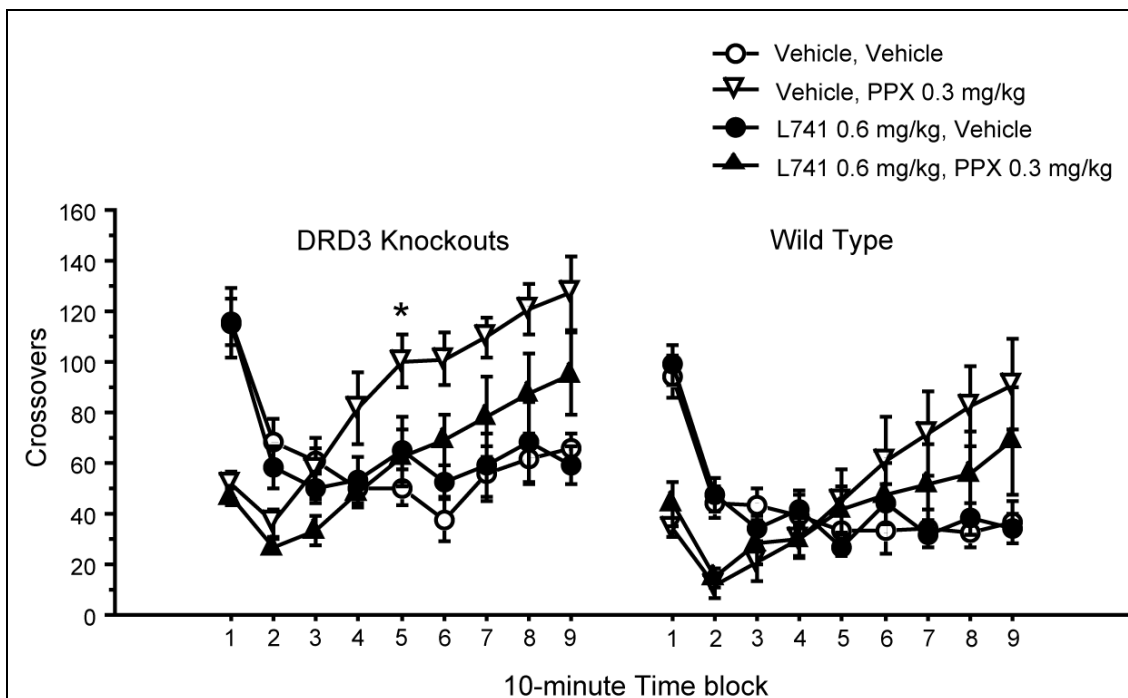
Figure 9.5: L741626 vs. PPX effects on locomotor activity in D3 KO and WT mice. Biphasic effects of PPX on locomotion are present in both KO and WT mice, though the hyperlocomotion phase begins 2 intervals earlier in KOs, preserving the 20-minute left-shift of PPX effects demonstrated in Fig. 9.4. Among KOs, there was a significant decrease in locomotor activity in mice receiving L741626 before PPX compared to mice receiving vehicle before PPX. There appears to be a selective antagonism of the later hyperlocomotion PPX effect in both WT and KO mice, while the earlier hypolocomotion effect is relatively spared. *$p<0.05$ for Vehicle-PPX compare to Vehicle-Vehicle.

E. Discussion:

Studies described in this report expand upon previous findings of PPX-induced alterations in locomotor and exploratory activity in unhabituated C57BL/6J mice by testing the dependence of this effect on D3 vs. D2 receptors. First, consistent with previous findings from a different laboratory and testing apparatus, PPX produced biphasic effects on locomotor activity in C57BL/6J mice (received from a vendor) across

90 min of observation (Chang et al., 2010a). A later hyperlocomotion effect was more pronounced in previous studies, potentially due to larger chamber dimensions or the presence of holes for measuring exploratory nose-pokes. As with vendor-acquired mice, WT mice bred within the present facility also exhibited this smaller PPX-hyperlocomotion effect. In all groups tested, higher doses of PPX resulted in a sustained hypolocomotion effect across 90 min of testing.

After establishing the reliability of this biphasic PPX effect, a cohort of WT and D3R mutant mice was tested for PPX-induced locomotor activity effects. Among unhabituated mice, no difference in basal locomotor activity was detected between D3 KO and WT mice. In other studies, basal (i.e. untreated or after saline injection) locomotor activity in both habituated and unhabituated D3 KO vs. WT mice has been either increased (Accili et al., 1996; Xu et al., 1997; Yarkov et al., 2010) or unchanged (Betancur et al., 2001; Karasinska et al., 2005; Pritchard et al., 2003; Risbrough et al., 2006; Waddington et al., 2001).

The biphasic vs. sustained locomotor effects of PPX 0.3 vs. 1.0 mg/kg, respectively, were also evident in D3 KO mice, suggesting that neither effect is fully dependent on D3 receptor activity. However, a D3 "signal" in the 0.3 mg/kg treatment group was suggested by a blunted and shorter-lived early hypolocomotion, and an enhanced and earlier-emerging hyperlocomotion in D3 KO vs. WT mice. This "release" of the PPX-induced hyperlocomotion in D3 KO mice may reflect a loss of a D3R opposition to D2R-mediated (L741626-sensitive) hyperlocomotion later in the session. Consistent with this interpretation, pharmacological, antisense knockdown, and D3R

mutant mouse studies have suggested that the D3R activation generally inhibits locomotor activity, acting in opposition to the behavioral effects of D1 and/or D2 receptor stimulation (cf. Holmes et al., 2004; Carr et al., 2002; De Boer et al., 1997; Ekman et al., 1998; McNamara et al., 2006; Ougazzal and Creese, 2000).

In addition to an apparent functional opposition of D3 vs. D2 systems, the present findings suggest that at least one other system must be contributing to PPX-mediated locomotor changes in mice. Thus, while L741626 significantly opposed PPX-induced hyperlocomotion in D3 mutants, and a similar pattern was detected in less activated WT mice, neither D3 receptor KO nor L741626 blocked the PPX-induced hypolocomotion. While D3 receptor activation appears to augment and sustain this early PPX-induced hypolocomotion (based on the blunting of this effect in D3 KOs), a prominent locomotor suppression is clearly detected in PPX-treated D3 mutants. One might argue that D2-stimulation by PPX has dual effects – suppressing forebrain DA release via D2 autoreceptors, and stimulating post-synaptic forebrain D2 receptors – and that while L741626 blocks both of these effects, it also imposes an independent locomotor suppression based on blockade of post-synaptic D2 receptors. Such an argument is made less compelling by the observation (Figure 9.5) that the dose of L741626 used in the present studies does not independently suppress locomotor activity in either WT or D3 mutant mice. Other receptor effects of PPX may provide a non-dopaminergic basis for a PPX-induced hypolocomotion that is neither D2- nor D3-dependent. Although PPX has very low binding affinity to all 5-HT receptor subtypes (cf. Kvernmo et al., 2006),

sustained administration of the drug has been found to alter the activity of dopamine, serotonin, and norepinephrine neurons (Chernoloz et al., 2009).

Our original studies of PPX effects in mice were designed to test a role for D3 receptors in the regulation of a different behavior: prepulse inhibition of startle (PPI). To our surprise, and in contrast to its effects in rats (Chang et al., 2010b; Weber et al., 2008a, 2009b) and humans (Swerdlow et al., 2009a), PPX had no significant effect on PPI in C57BL/6 mice, despite the fact that 1) PPI was potently disrupted by the mixed DA agonist, apomorphine; and 2) PPX was clearly bioactive in these mice, based on the patterns of locomotor activity that were replicated and extended herein (Chang et al., 2010a). One possible explanation for these findings is that, in the doses studied, PPX is both D2- and D3- "inert", i.e.. has no detectable effects on D2 or D3 systems in C57BL/6 mice; alternatively, PPX might be D2- and D3-active in these mice, but PPI might not be sensitive to these effects. The present study provides some evidence that PPX produces both a "D3 signal" (based on the apparent "release" of D2-opposing locomotor effects in D3 KO mice) and a "D2 signal" (L741626-sensitive hyperlocomotion) in C57BL/6 mice. Thus, unlike rats − in which PPX and other D2- and D3-preferential agonists potently modify PPI (cf. Swerdlow et al., 2008b; Caine et al., 1995; Swerdlow et al., 1998a; Varty and Higgins, 1998; Weber et al., 2008a, 2009b; Zhang et al., 2007) − the present findings are consistent with the hypothesis that neither of these D2-family receptors potently regulate sensorimotor gating in mice (Ralph-Williams et al., 2003; Ralph and Caine, 2005; Chang et al., 2010a). The *in vivo* selectivity of effects on rodent behavior for many preferential D3 agonists remains controversial. Still, by combining genetic and

pharmacological tools, the present study identified separable D2- and D3-mediated locomotor "signals" of PPX in mice, and extends previous findings (Chang et al., 2010a) by suggesting that neither of these systems contribute to the regulation of PPI in C57BL/6 mice.

CHAPTER 10:

Disparate effects of pramipexole on locomotor activity

and sensorimotor gating in Sprague Dawley rats

Wei-li Chang, Michelle R. Breier, Alex Yang, Neal R. Swerdlow

A. Abstract:

Prepulse inhibition (PPI) of acoustic startle and locomotor activity are both widely studied in the preclinical development of dopaminergic agents, including those acting at D3 dopamine receptors. In mice, the dopamine D3 receptor-preferential agonist pramipexole (PPX) alters locomotor activity in a biphasic manner at doses that have no effect on PPI. The present study examined the time-course of PPX effects on locomotion and PPI in rats. In adult male Sprague-Dawley rats, PPX (0, 0.1, 0.3, 1.0 mg/kg) was injected prior to measurement of locomotor activity for 90 min in photobeam chambers. Based on disparate early vs. late effects of PPX on locomotion, the effects of PPX (0 vs. 0.3 mg/kg) on PPI were tested 20 and 80 min after injection. All doses of PPX decreased locomotor activity for 30 min compared to vehicle, and the higher doses stimulated hyperlocomotion later in the session; the late hyperlocomotion, but not the early hypolocomotion, was blocked by the D2-selective antagonist, L741626 (1.0 mg/kg sc). In contrast to its locomotor effects, PPX caused a similar reduction in PPI at 20 and 80 min after administration. These findings suggest both a temporal and pharmacological dissociation between PPX effects on locomotor activity and PPI; these two behavioral

measures contribute non-redundant information to the investigation of D3-related behavioral pharmacology.

B. Introduction:

The dopamine D3 receptor has been studied as a potential source of pathology or target for novel pharmacotherapeutics for several neuropsychiatric disorders, including schizophrenia, Tourette Syndrome, and drug addiction (cf. Sokoloff et al., 2006; Weber et al., 2009b). Animal models can help elucidate the mechanisms by which activity at D3 receptors regulates behaviors of relevance to these disorders. Two common behavioral measures in animal models of neuropsychiatric disorders are prepulse inhibition of startle (PPI) and locomotor exploration.

We (Chang et al., 2010b; Swerdlow et al., 2009a; Weber et al., 2008a, 2009b) and others (Caine et al., 1995; Varty et al., 1998; Zhang et al., 2007) have reported that D3-preferential agonists can reliably suppress PPI in rats. These effects are detected across multiple rat strains, using different stimulus modalities, in males and females, and after either systemic or intracerebral administration (Chang et al., 2010b; Weber et al, 2010a). In contrast to their effects in rats, many D3-preferential and mixed D2/D3 agonists have no effects on PPI in mice (Chang et al., 2010a; Ralph and Caine, 2005; Ralph-Williams et al., 2003), consistent with other reports that different dopamine receptor subtypes regulate PPI in rats vs. mice (Ralph et al., 1999). Genetic approaches using mice are therefore suboptimal for elucidating the molecular mechanisms underlying the D3

regulation of sensorimotor gating. For example, we reported previously that the D3-preferential agonist, pramipexole (PPX), disrupts PPI in rats but not in C57BL/6J mice, a common background strain knockout studies; in contrast, PPI is disrupted in both species by the mixed D1/D2 agonist, apomorphine (Breier et al., 2010; Caine et al., 1995; Caldwell et al., 2009; Chang et al., 2010a; Martin et al., 2008; Ralph-Williams et al., 2002, 2003; Russig et al., 2004; Semenova et al., 2008; Swerdlow et al., 2005, 2008a; Van den Buuse et al., 2005; Weber et al., 2008a, 2008b, 2009a; Yee et al., 2004). Interestingly, in mice, PPX produces dose-dependent, biphasic changes in locomotor exploratory activity, consisting of an early locomotor suppression followed by a later, D2-dependent hyperactivity (Chang et al., 2010a).

Rather than pursue separate, parallel efforts to elucidate the biology of two different D3-mediated effects (on locomotor activity and PPI) in two different species (mouse and rat, respectively), we tested PPX effects on both locomotor activity and PPI in rats, with the goal of developing a simpler, single-species model for understanding D3-mediated effects on these behavioral measures.

C. Methods:

1. Animals: Adult male Sprague-Dawley rats (n=40, 225-250 g; Harlan Laboratories, Livermore, CA) were housed 2-3 animals per cage and maintained on a reversed light/dark schedule with food and water available *ad libitum*. All testing occurred during the dark phase, and rats were handled within 2 d of arrival and allowed

to acclimate for at least 7 days before behavioral testing. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, revised 1985) and were approved by the UCSD Animal Subjects Committee (protocol #S01221).

2. Drugs: PPX was obtained from Toronto Research Chemicals (North York, Ontario, Canada) and L741626 from Tocris (Ellisville, MO, USA). PPX (0, 0.1, 0.3, 1.0 mg/kg) was dissolved in saline and injected subcutaneously. L741626 (0, 1.0 mg/kg) was dissolved in 0.15% lactic acid/water (w/v) and pH was adjusted to ≥ 5 with NaOH; injection was subcutaneous. PPX was administered immediately before locomotor testing, and either 15 or 75 min before placement into startle chambers for PPI testing. For locomotor studies using D2 antagonist pretreatment, L741626 was injected 30 min before PPX injection and placement in activity chambers.

3. Locomotor testing: Locomotor activity was measured using wire-mesh photocell cages (22×35×15 cm) fitted with two parallel infrared beams 1 cm above the floor, perpendicular to the long axis of the cage. The total number of beam breaks and crossovers (sequential interruption of separate beams) was calculated for each 10 min interval during 90 min of testing; rats were not habituated to the test chamber before locomotor activity measurement began. To understand the behavioral basis for changes in photocell activity counts, animals were observed through a viewing window and assessed

for the presence of specific behaviors (Fray et al., 1980) by an individual who was blind to their drug condition; any combination of behaviors could be present during each rating period. Animals were first tested (test day 1) with either vehicle or active dose of agonist, and then assigned to new dose groups balanced for previous PPX doses, for studies of antagonist/agonist combinations (test days 2 and 3), with 7-9 days between test days.

4. Prepulse Inhibition Testing: Startle chambers (San Diego Instruments, San Diego, CA, USA) consisted of a Plexiglas cylinder 8.2 cm in diameter resting on a 12.5 × 25.5 cm Plexiglas frame within a ventilated enclosure, housed in a sound-attenuated room. Noise bursts were presented from a speaker mounted 24 cm above the cylinder, and a piezoelectric accelerometer mounted below the Plexiglas frame detected and transduced motions from within the cylinder. SR-LAB microcomputer and interface assembly controlled stimulus delivery and digitized (0-4095), rectified, and recorded stabilimeter readings. One hundred 1-ms readings were collected beginning at stimulus onset and averaged to yield the startle amplitude.

PPI effects were tested in a separate group of rats. Before drug testing, rats were exposed to a short "matching" session in startle chambers, which consisted of a 5 min acclimation period with 70 dB(A) background noise and then 17 PULSE-ALONE trials (40 ms – 120 dB(A) noise bursts) interspersed with 3 PREPULSE+PULSE trials in which PULSE-ALONE was preceded 100 ms (onset-to-onset) by a 20 ms noise burst, 12 dB

above background. The average %PPI from this session was used to assign rats to drug/dose groups with matched baseline PPI.

Based on findings from locomotor studies, PPI studies utilized pretreatment time as a between-subject factor, and vehicle vs. drug as a within-subject factor, with balanced dose orders. Test days were 5 days apart. 15 or 75 min after PPX (0, 0.3 mg/kg) injection, rats were placed into startle chambers for a 5 min acclimation period with a 70 dB(A) background noise. Active trials were presented in pseudorandom order and included: (1) PULSE-ALONE (40 ms – 120 dB(A) noise burst); (2-4) PREPULSE+PULSE (PULSE-ALONE preceded 100 ms (onset-to-onset) by a 20 ms noise burst either 5, 10, or 15 dB above background). Interspersed between active trials were NOSTIM trials in which no stimulus was presented but activity was recorded. Average ITI between active trials was 15 s. Session duration was 18.5 min, including the acclimation period.

5. Data Analysis: For locomotor data, photocell beam break and crossover counts were analyzed by ANOVA. Comparable results were found for both beam breaks and crossovers, so crossovers are presented to avoid redundancy. Data are reported as group mean ± SEM of total crossovers for each interval. On Day 1 of locomotor testing, PPX dose was a between-subject factor. For Days 2 and 3, L741626 pretreatment was the between-subject factor and PPX was a within-subject factor.

Behavioral ratings were scored as 'present' or 'absent'. Analyses for behavioral rating data were based on those described in Fray et al. (1980). Briefly, the percentage of

rats within each dose and interval displaying behaviors from each category was entered into a contingency table, which was then assessed for heterogeneity using a likelihood ratio method, called the 'information statistic' (Kullback, 1968; Robbins 1977), which is analogous to the χ^2 but is not constrained by small cell frequencies. Calculation of 2Î is detailed in Fray et al. (1980).

PPI was defined as 100-[(startle amplitude on PREPULSE+PULSE trials/startle amplitude on PULSE-ALONE trials) × 100], and was analyzed by mixed design ANOVAs. Data was inspected for "non-responders", defined by mean startle response to PULSE-ALONE trials of less than 10. There was one non-responder on the second day of testing, who was eliminated from all analyses. Repeated measures ANOVAs were used to assess responses to PULSE-ALONE, PREPULSE+PULSE, or NOSTIM trials.

For all photocell and PPI studies, relevant post-hoc comparisons were conducted using Fisher's PLSD and one-factor ANOVA tests, and alpha was set at 0.05.

D. Results:

1. PPX effects on locomotor activity: Rats exhibited a dose-dependent, biphasic pattern of PPX-induced locomotor changes that was strikingly similar to that previously observed in mice (Chang et al., 2010a). Repeated measures ANOVA for crossovers revealed significant effects of PPX dose (F=5.23, *df* 3,20; *p*<0.01) and interval (F=9.75, *df* 8,160; *p*<0.0001), and a PPX × interval interaction (F=8.83, *df* 24,160; *p*<0.0001; Fig.

10.1). Post hoc testing showed that crossovers for all active doses were significantly different from the vehicle-treated group during the first 30 min of testing, and that the 0.3 mg/kg PPX group had significantly increased crossovers during the last 40 min of testing. Inspection of the data (Fig. 10.1) suggested that there was also an increase in crossovers in the PPX 1.0 mg/kg group, but this effect was only statistically significant during Interval 7.

Understanding the basis for group differences in photocell counts requires information about the types of behaviors being exhibited by the rats. For example, reduced photocell counts can result from either sedation or intense stereotypy. Data (Fig. 10.2) revealed that the early PPX-induced hypoactivity was not accompanied by any stereotyped behaviors; behaviors during later periods of elevated photocell activity included exploratory locomotion, rearing, and sniffing.

Figure 10.1: Dose-response effects of PPX on locomotor activity in rats. PPX (0, 0.1, 0.3, 1.0 mg/kg) was injected immediately before animals were placed into photobeam chambers. Crossovers were measured in 10-minute intervals across 90 minutes. Locomotor activity was significantly reduced compared to vehicle-treated rats in all active PPX dose-groups during the first 30 min. Rats treated with 0.3 mg/kg demonstrated increased locomotor activity in the last 40 min; hyperlocomotion in the 1.0 mg/kg dose group was only significant during the 60-70 min interval. *p<0.05, *n*=6 rats/dose group.

Figure 10.2: PPX dose-response effects on behavioral ratings during locomotor activity testing. Animals were observed briefly during each 10-min interval of locomotor testing for the presence of behaviors (as described in Fray et al. (1980)). Data points represent the percentage of rats from that dose group that exhibited behavior from each category. Boxes above data points indicate statistical significance ($p<0.05$) within each interval of that particular dose group compared to lower doses. Absence of boxes indicates a lack of overall significance for that interval. A change in box color from white to black or from black to white signifies a statistical difference between corresponding dose groups; no color change indicates no statistical difference. For example, in Interval 3 of SNIFFING, 0.1 mg/kg PPX is statistically different from 0 mg/kg PPX. 0.3 mg/kg PPX is not different from 0.1, but 1.0 mg/kg is different from 0.1 and 0.3 combined. Intense stereotyped behaviors – gnawing, licking, head down, grooming – were not observed in more than one rat per dose, at any interval. *n*=6 rats/dose group

% of Rats

STILL

SNIFFING

LOCO

REARING

10-minute Time Block

Saline
PPX 0.1 mg/kg
PPX 0.3 mg/kg
PPX 1.0 mg/kg
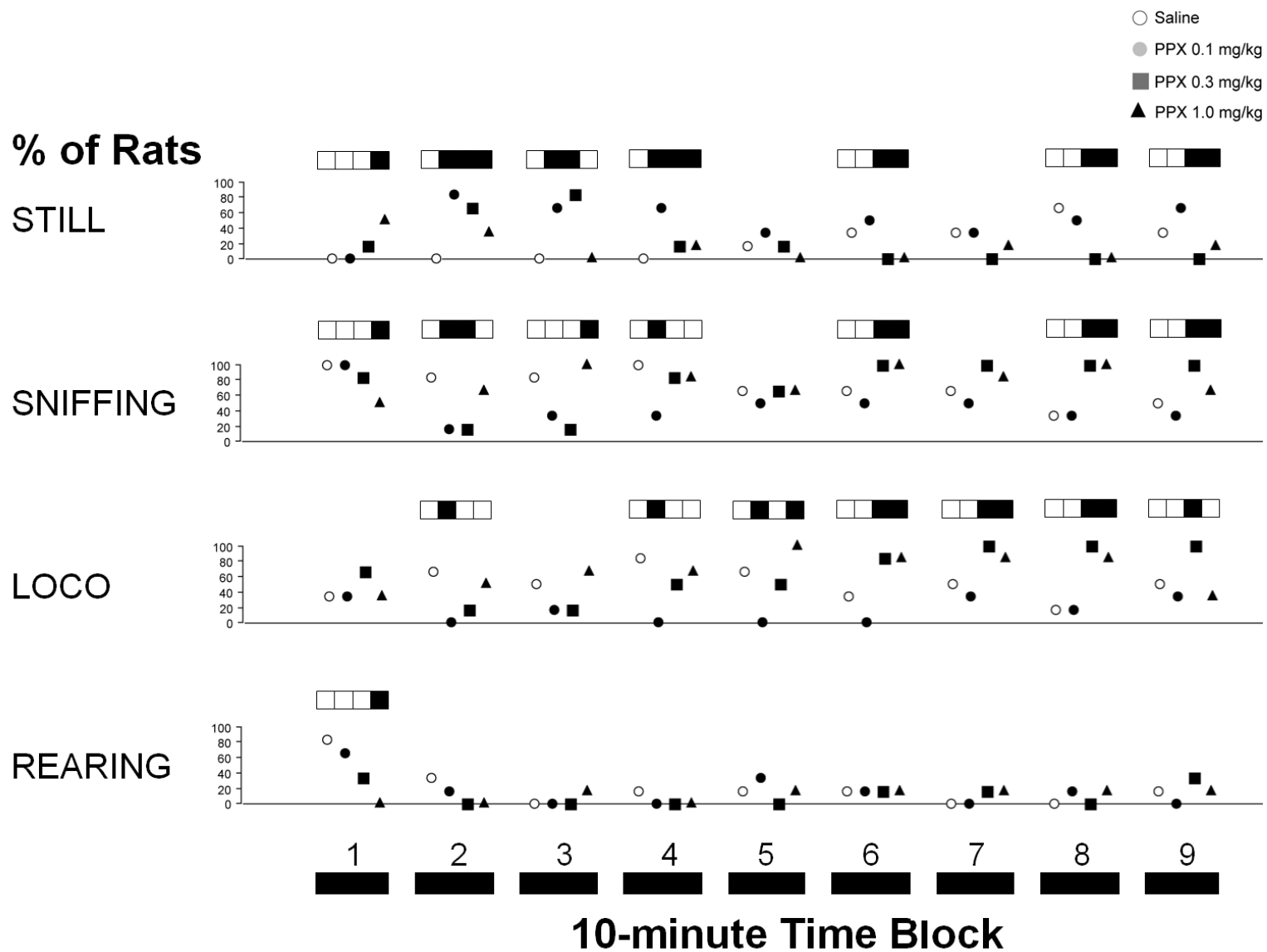
2. Effect of D2 blockade on PPX-induced locomotor activity: Animals were assigned to new dose groups, and tested for locomotor activity with L741626 pretreatment (0, 1.0 mg/kg) before PPX injection (0, 0.3 mg/kg). This dose of L741626 was chosen based on our experience that it is "D2-selective", e.g. opposes the effects of D2 agonists but not PPX on PPI in SD rats (Weber et al., 2009b, 2010b); the dose of PPX was chosen so that we could assess the impact of D2 blockade on both PPX-induced hypo- and hyperactivity (Fig. 3). Repeated measures ANOVA revealed significant effects of L741626 pretreatment (F=23.38, df 1,14; $p<0.0005$) and interval (F=18.05, df 8,112; $p<0.0001$), and significant interactions of L741626 pretreatment × interval (F=7.30, df 8,112; $p<0.0001$), PPX × interval (F=22.11, df 8,112; $p<0.0001$), and L741626 × PPX × interval (F=10.83, df 8,112; $p<0.0001$). Similar to what was detected on test day 1 (Fig. 10.1), among rats receiving the vehicle pretreatment, 0.3 mg/kg PPX led to an early hypoactivity during the first 30 min, followed by significant hyperactivity during the last 30 min of testing. Pretreatment with the active dose of L741626 (1.0 mg/kg) did not affect the early PPX-induced hypoactivity, but completely blocked the late PPX-induced hyperactivity (see Fig. 10.3 for specific post-hoc comparisons).

Figure 10.3: Effect of L741626 on biphasic locomotor response to PPX. L741626 (0, 1.0 mg/kg) was injected 30 min prior to PPX (0, 0.3 mg/kg) and placement of animals into photobeam chambers. As in Fig. 1, PPX produced decreased and then increase locomotor activity compared to vehicle. The late hyperlocomotion effect was blocked by L741626 pretreatment, while early hypolocomotion was unaffected. *$p<0.05$ for Vehicle vs. PPX in animals that did not receive active pretreatment (open circle vs. open triangle). #$p<0.05$ for Vehicle vs. L741626 pretreatment in animals that received PPX injection (open triangle vs. closed triangle).

3. PPX effects on PPI: The effects of PPX on PPI were tested using both the dose (0.3 mg/kg) and time points (20 and 80 min post-injection) associated with the biphasic effects of PPX on locomotor activity. In contrast to its effects on locomotor activity, PPX-induced changes in PPI were constant rather than biphasic (Fig. 10.4A). Repeated measures ANOVA revealed significant effects of PPX dose (F=54.96, *df* 1,13; *p*<0.0001) and prepulse intensity (F=15.78, *df* 2,26; *p*<0.0001), but no effect of pretreatment time (F<1). There were no significant 2-way interactions or meaningful 3-way interactions; when collapsed across intensities, there was a significant effect of PPX on PPI at both 20 and 80 min pretreatment times, and PPI did not differ between the two PPX-treated groups. PPX also had a significant effect on startle magnitude (F=6.71, *df* 1,13; *p*<0.03) (Fig. 10.4C), but there was no effect of pretreatment time (F<1) on startle, and no significant interactions. Splitting the 20 and 80 min pretreatment groups at the median for PPX-induced startle suppression (startle magnitude on vehicle treatment day minus startle on PPX treatment day), there was no significant interaction between low or high startle suppression and PPX, and no other meaningful interactions with startle suppression. Although PPX appeared to slightly increase NOSTIM activity, this effect did not reach statistical significance (main effects of PPX and time, and interactions all NS; Fig. 10.4D).

Figure 10.4: Effects of PPX 0.3 mg/kg on PPI, startle, and NOSTIM activity at time points corresponding to hypo- and hyper-locomotion effects. A different set of rats were tested with the key dose of PPX with either a 15 or 75 min pretreatment time before PPI testing. [A]: Effects on %PPI. Data are collapsed across prepulse intensities. $p<0.006$ for Vehicle vs. PPX at each pretreatment time. [B]: Locomotor activity data for this PPX dose as shown in Fig. 10.1. Gray boxes indicate the post-injection intervals corresponding to these PPI test sessions. [C]: Effects on startle magnitude. [D]: Effects on NOSTIM activity. Note the scale of NOSTIM activity magnitude relative to the scale for pulse-elicited startle responses.

E. Discussion:

Taken together with our previous findings using a similar testing paradigm in mice, the present findings support several conclusions. First, the impact of PPX on

locomotor activity in SD rats is strikingly similar to that in C57BL/6J mice (Chang et al., 2010a), in terms of dose sensitivity and time course, including the elicitation of biphasic hypo- and hyperlocomotion. While this does not prove that similar underlying mechanisms mediate these PPX effects across species, it demonstrates that some mechanisms for both PPX-mediated reductions and increases in exploratory behavior exist in both mice and rats, that exhibit nearly-identical dose- and time-sensitivities. Second, in rats and mice, the late PPX-induced hyperactivity is blocked by L741626, suggesting that it is a D2-dependent drug effect in both species. Third, in both species, the early PPX-induced hypoactivity is not antagonized - and is perhaps potentiated - by D2 blockade. This pattern would be consistent with a D3-mediated stimulation of presynaptic receptors, resulting in a reduction of DA release. While many other mechanisms might be involved, such an effect of presynaptic D3 activation might be expected to produce the observed patterns: it would not be antagonized by D2 blockade, and might add to or synergize with the effects with L741626 (although, as in the present study, activity "floor effects" during this early PPX-induced hypoactivity might complicate the detection of such additive or synergistic effects). Direct evidence for such an autoreceptor effect is not compelling; in fact, microdialysis findings suggest that striatal DA release is not altered during the first two hours after PPX administration (Lagos et al., 1998). Also arguing against an exclusive role of D3 autoreceptors in this early hypoactivity, at least in mice, is our finding that PPX-induced hypoactivity is present - though diminished and shorter-lived - in mutant mice lacking D3 receptors (Richtand et al., 2011).

Across previous reports of PPX effects on locomotor activity in rats, the same dose-range of PPX has yielded very different dose-response properties (Kitagawa et al., 2009; Lagos et al., 1998; Maj et al., 1997; Svensson et al., 1994a). Specifically, a "U-shaped" dose-response curve, characterized by decreased locomotion at lower doses and increased locomotion at higher doses, is reported when testing is conducted 1-2.5 hours after PPX injection (Lagos et al., 1998; Maj et al., 1997), while hypolocomotion at all doses is seen with shorter wait times of 30 min or less (Kitagawa et al., 2009; Lagos et al., 1998; Svensson et al., 1994a). One report describes a similar, time-dependent biphasic effect with 0.5 mg/kg PPX eliciting decreased locomotion at 0-30 min post-injection and increased locomotion at 120-150 min post-injection (Lagos et al., 1998). The present results confirm that different time intervals between PPX administration and activity measurements capture mechanistically different effects of PPX: D2-independent early hypo-locomotion and D2-dependent later hyper-locomotor effects of a single dose of PPX were detected via an extended measurement period, divided into shorter measurement intervals. Conceivably, U-shaped dose-response effects of other D3-preferential agonists might also reflect a temporal conflation of disparate receptor mechanisms (Ahlenius and Salmi, 1994; Collins et al., 2007; Khroyan et al., 1997; Millan et al., 2004b; Pugsley et al., 1995; Svensson et al., 1994b).

Having detected apparently multi-mechanism, biphasic locomotor effects of PPX, it was next possible to ask whether the PPI-disruptive effects of PPX exhibited such a dynamic profile in rats; this was not possible in mice, due to their complete insensitivity to PPX-induced PPI changes (Chang et al., 2010a). Most PPX effects on PPI have been

studied 15-45 min post-injection (Chang et al., 2010b, Swerdlow et al., 2009a; Weber et al., 2008a, 2009b). In the present study, we determined that the PPI-disruptive effects of PPX were identical, whether tested 20 min after injection, coinciding with the D2-insensitive PPX-induced hypoactivity, or 80 min after injection, when PPX produced a D2-dependent hyperactivity. Thus, there is no apparent time-sensitive "shift" in the systems mediating the PPI-disruptive effects of PPX, and by extension, these PPI-disruptive effects appear to be independent of at least one (and perhaps both) of the mechanisms regulating PPX-induced changes in locomotor activity. That the early PPI-disruptive effects of PPX are relatively insensitive to L741626 (and are certainly not additive or synergistic with such effects) (Weber et al., 2009b), suggests that they are neither D2-dependent, nor do they reflect DA-suppressive effects that might result from activation of D3 autoreceptors. Sensitivity of the late PPI-disruptive effects of PPX to L741626 has not yet been tested; because no obvious temporal shift was detected in PPX effects on PPI or other startle measures, there is no clear reason to suspect a greater sensitivity to D2 blockade in the late vs. early PPI-disruptive effects of PPX.

In summary, the effects of PPX on locomotor activity in rats - as with those previously detected in mice - are most easily explained by an early hypodopaminergic response that includes (but is not exclusively mediated by) a participation of D3 autoreceptors, and a late hyperdopaminergic response reflecting the activation of D2 receptors. In contrast, the species-specific PPI-disruptive effects of PPX appear to be distinct from at least one and perhaps both of these mechanisms. Consequently, these two behavioral measures contribute non-redundant information about the neurobiological

effects of D3 receptor activation, and its potential contributions to the etiology or treatment of brain disorders.

F. Acknowledgements:

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CHAPTER 11:

Technical note:

In vivo suppression of dopamine D3 receptor expression

in adult rats using lentiviral vectors

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Marie, Neal R. Swerdlow

A. Abstract:

1. Background: Virtually all approved medications for schizophrenia act at dopamine (DA) receptors, antagonizing the D2 and D3 DA receptor subtypes in a nonselective manner. D3-selective compounds may represent a novel class of antipsychotics that lack the major side effects of non-selective antagonists. Prepulse inhibition (PPI) of the acoustic startle reflex, an operational measure of sensorimotor gating, can be modified by DA-active drugs in humans and rodents, and is used in rodents as a preclinical model to predict antipsychotic potential. Unfortunately, many drugs that act on D3 receptors do not affect PPI in mice, and thus traditional "knockout" strategies cannot be used to assess the *in vivo* receptor subtype specificity of drugs that display preferential binding to D3 receptors *in vitro*. This study aims to use viral vectors to suppress D3 receptor expression in the adult rat brain for the purpose of testing receptor specificity of DA-active drugs in awake behaving animals.

2. Methods: A pseudotyped lentiviral vector packaging system is used to deliver one of three plasmids containing sequences for small-hairpin RNAs (shRNA) that have been shown to successfully silence D3R expression in the rat brain or a CMV-eGFP expression cassette (Control condition). Virus containing one of 4 different constructs is infused at a high titer into the nucleus accumbens core (NAcC) bilaterally (1 μl/side at 0.2 μl/min). Adult male Sprague-Dawley rats are tested for PPI prior to injection surgery and again on the day of tissue collection. Successful infection by lentivirus vector is assessed by Western blot or gross visualization of GFP in brain tissue collected from vehicle group. D3 receptor expression is measured at two-week intervals 2 to 10 weeks after lentivirus infusion using either Western blot or quantitative rt-PCR.

3. Results: Two weeks after lentiviral infusion, GFP expression can be seen in the NAc with Western blot. Microscopy of GFP demonstrated the anatomical extent of lentiviral uptake in the region of the accumbens. Evidence of some D3 receptor suppression were detected by Western blot at 4 weeks and rt-PCR at 6 weeks, but these effects were not observed at other time points and were not replicated in other test cohorts. Expected patterns of startle modulation were detected across groups at all time points, including significant reflex habituation and intensity-dependent prepulse inhibition, but levels of startle magnitude, habituation and PPI did not differ significantly between Control and active D3-shRNA constructs. Additionally, no behavioral differences were observed in Control vs. D3-shRNA infused rats in behavioral response to PPX.

4. Conclusions: Lentiviral injections into the nucleus accumbens appeared to yield anatomically localized uptake and expression of vector-delivered genetic material in the Control condition, but no reliable and replicable evidence of D3 receptor suppression was demonstrated. Behavioral testing likewise did not detect evidence of bioactivity of D3-shRNA. The present findings suggest that lentiviral vector-mediated suppression of D3 receptor expression in brain regions relevant to sensorimotor gating is currently not a feasible method of assessing the effects of selective D3 receptor compounds.

B. Introduction:

Prepulse inhibition (PPI) of acoustic startle is disrupted in several disorders, including schizophrenia. PPI deficits can be induced in rats by mixed DA agonists like apomorphine (APO) (Swerdlow et al., 1986) and the preferential DRD3 agonist pramipexole (PPX) (Chang et al., 2010b, Swerdlow et al., 2009a; Weber et al 2008a, 2009b). Until recently, there have been relatively few compounds that display selectivity for D3 over D2 receptors *in vivo*. PPX has been reported to have D3:D2 binding preference ranging from 7:1 to 160:1 *in* vitro (Millan et al., 2002; Piercey et al., 1996; Svensson et al., 1994a), and along with the D3-preferential agonist ropinirole, has both novel therapeutic value in treating specific movement disorders, and a novel liability profile in inducing specific behavioral syndromes characterized by high hedonic or novelty valence (cf. Ahlskog, 2011).

While pharmacological approaches to testing for DRD3 selectivity have been informative, molecular manipulation of DRD3 expression would provide more definitive evidence of D3 vs. D2 selectivity in awake behaving animals. However, we and others have previously shown that the D3 preferential and D2/D3 agonists do not affect PPI in mice (Chang et al., 2010a, Ralph-Williams et al., 2003; Ralph and Caine, 2005), precluding the usefulness of DRD3 knockout mice to answer these questions. Here, we aim to "knock down" D3 receptor expression in the adult rat brain using viral vector delivery of D3-shRNA, with the eventual goal of testing receptor specificity of DA-active drugs that alter PPI.

C. Methods:

1. Experimental Animals: Adult male Sprague-Dawley (n = 78, 225-250 g; Harlan Laboratories, Livermore, CA) rats were housed 2-3 animals per cage, and maintained on a reversed light/dark schedule with water and food available *ad libitum.* Rats were handled within 2 d of arrival. Surgery occurred between 10-16 days after arrival. Testing occurred during the dark phase. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, revised 1985) and were approved by the UCSD Animal Subjects Committee (protocol #S01221).

2. Behavioral testing: Rats were assigned to lentivirus groups based on %PPI in a matching session. Testing before and after lentivirus infusion began with 5 min of acclimation and then 120 dB(A) pulse trials of 40 ms duration that were either presented alone (PULSE-ALONE), or preceded 100 ms (onset to onset) by a 20 ms prepulse of either 5, 10, or 15 dB above background. Average ITI between active trials was 15 s.

3. Lentivirus: Three plasmids containing sequences for small-hairpin RNAs that have been shown to successfully silence DRD3 expression in the rat brain (Bahi et al., 2005) have been obtained through a generous gift from Dr. Jean-Luc Dreyer. Plasmids containing Lenti-D3-shRNA1, Lenti-D3-shRNA2, and Lenti-D3-shRNA3 were transformed into NEB 5-alpha competent *E. coli*. A large scale preparation of plasmids was conducted according to the protocol from QIAGEN EndoFree Plasmid Maxi Kit. Ultrapure plasmid DNA will be eluted in high-salt buffer and precipitated by isopropanol. The three Lenti-D3-shRNA plasmids were sequenced by Retrogen, Inc. to confirm amplification of the correct constructs. Custom lentivirus vector production was performed by the Salk Viral Vector Core using procedures adapted from (Tiscornia et al., 2006a, 2006b). Briefly, 293T cells were transfected with shRNA-expressing pTK431 vector plasmids, helper plasmid psPAX2, and the VSV.G expression plasmid pMD2.G; virus was then purified by ultracentrifugation on a sucrose cushion. Medium containing replication-deficient lentiviral particles were harvested twice and pooled before filtration and concentration. Concentrated virus was resuspended in HBSS and stored at -80˚C in 10 µl aliquots. Custom lentiviral preps were titrated by serial dilution and infection of 293T cells, using

qPCR to detect infection titer in transforming units (TU) per ml. The final product was replication-incompetent shRNA-expressing second-generation lentivirus.

Lentivirus constructs were injected during stereotaxic surgery (below). Surgeries were performed in three sets of rats: in Exp. 1 and 2, each rat received one of three D3-shRNA constructs or the Lenti-CMV-eGFP control at a titer of 2×10^9 IU/ml. For Exp. 3, animals received either a "cocktail" of all three Lenti-D3-shRNA at ($3-5.4\times10^{10}$ IU/ml) or Lenti-CMV-eGFP (1×10^{10} IU/ml). In all experiments, 1μl of virus per side was infused at 0.2 μl/min via a motorized microsyringe. For the Exp. 1 and 2, one rat from each group was sacrificed every other week starting at Week 2 for either Western blot (Exp. 1) or quantitative rt-PCR (Exp. 3) analyses of D3 receptor expression. In Exp. 3, six rats from the Active and Control lentivirus groups were sacrificed at weeks 2, 4, and 6 post-infusion and assayed for DRD3 expression in the NAC with quantitative rt-PCR.

4. Surgery: Rats received 0.1 ml atropine sulfate (Vedeo, 0.054 mg/ml sc) 15-30 min before being anesthetized with sodium pentobarbital (Abbott, 60.0 mg/kg/ml ip). Fully anesthetized rats were placed in a Kopf stereotaxic instrument (tooth bar at -3.3 mm). Lentivirus was infused into the nucleus accumbens core (NAcC) (AP +1.2, L ±1.7, DV -4.3 (Bregma); Fig. 11.1) through 30 ga needles attached to PE 10 tubing over 5 minutes. Rats were isolated in Biosafety Level 2 facilities for 3 days after infusion and monitored daily post-operatively.
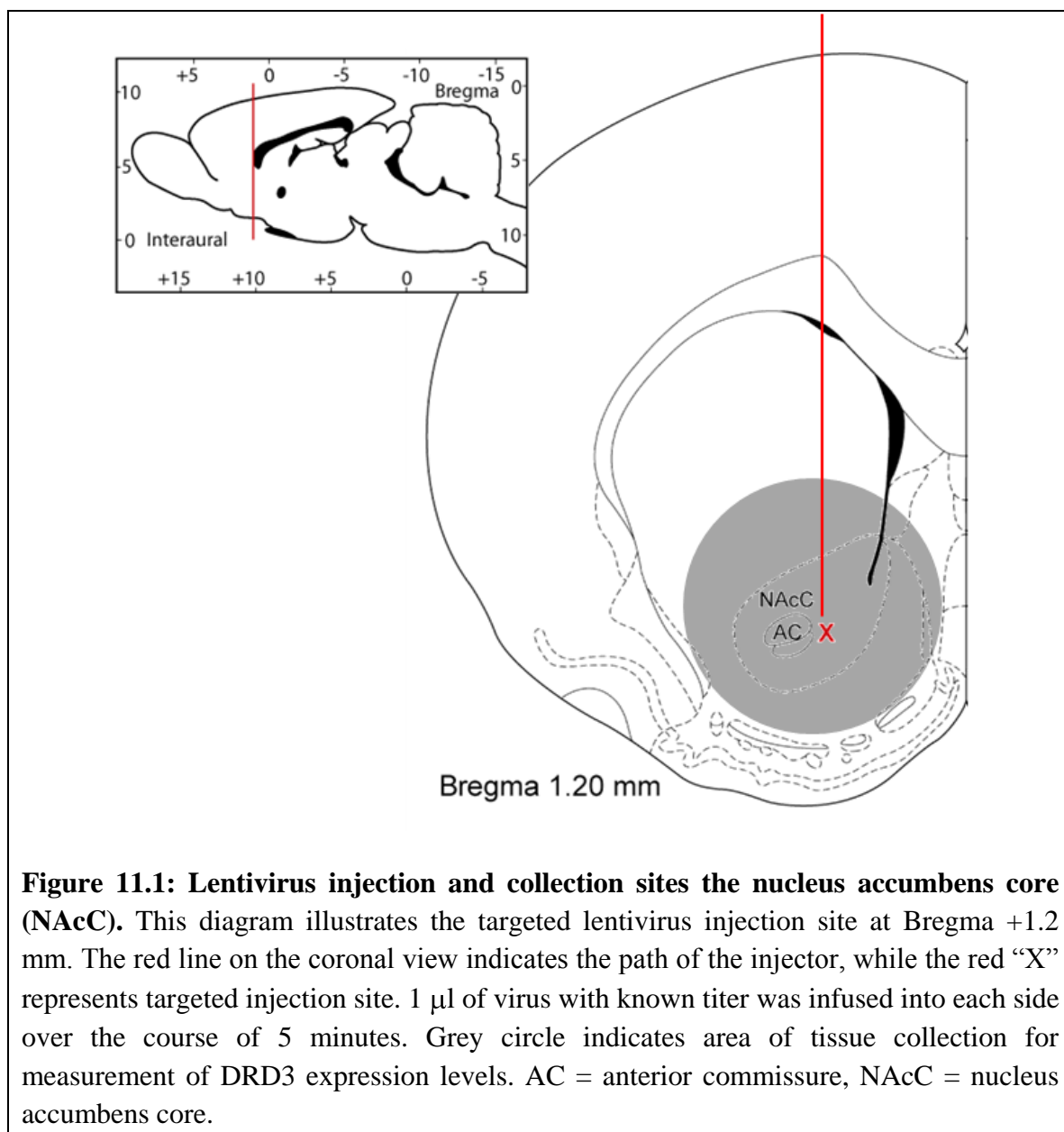
Figure 11.1: Lentivirus injection and collection sites the nucleus accumbens core (NAcC). This diagram illustrates the targeted lentivirus injection site at Bregma +1.2 mm. The red line on the coronal view indicates the path of the injector, while the red "X" represents targeted injection site. 1 µl of virus with known titer was infused into each side over the course of 5 minutes. Grey circle indicates area of tissue collection for measurement of DRD3 expression levels. AC = anterior commissure, NAcC = nucleus accumbens core.

3. Western blot: Rats were decapitated and brains rapidly dissected. NAc samples were obtained with a 2 mm diameter punch from 1 mm thick coronal slabs (Bregma +1.2 to +2.2 mm; Fig. 11.1) and flash frozen. Tissue was homogenized, subjected to denaturing NuPAGE Bis-Tris gel electrophoresis, and transferred to

nitrocellulose membranes. Membranes were incubated with antibody to DRD3 (Abcam, Santa Cruz) or β-actin (Sigma). The intensity of immunoreactive bands are quantified using NIH ImageJ, and immunopositive bands normalized relative to β-actin. Suppression of DRD3 expression in Lenti-shRNA injected rats is expressed as a percentage of DRD3 expression in Control (Lenti-CMV-GFP) rats.

4. Quantitative rt-PCR: NAc samples are collected as detailed above for Western blot, but tissue was placed immediately into RNALater (Ambion) and onto dry ice. Samples were stored at -80˚C until RNA extraction with QIAGEN RNeasy Mini Kit. Quantitative rt-PCR was performed by UCSD Center for Aids Research Genomic Core. On-column DNAse digestion was performed with the QIAGEN RNase-free DNase set, and cDNA was made with qScript cDNA SuperMix (Quanta Biosciences). qPCR was conducted on Applied Biosystems 7900HT Fast Real-Time PCR System. Standard curves for DRD3 (Applied Biosystems) expression were made with one rat from the Control group; all samples were run in duplicate and normalized to GAPDH (Applied Biosystems) expression levels. Data are expressed as a fold change from or percentage of average DRD3 expression in the Control rats.

5. Perfusion: Additional rats that received stereotaxic lentivirus injection in Exp. 2 were perfused for visualization of GFP. Rats were anesthetized and then perfused with a buffered (pH 7.4) 4% solution of para-formaldehyde. Dissected brains were frozen

sectioned in the transverse plane at 40 μm. Sections were wet mounted for light and fluorescence microscopy.

D. Results:

1. Molecular Results: Successful infection of the NAc was confirmed with both fluorescence microscopy and WB for GFP expression (Fig. 11.2). The image in Fig. 11.2B suggests that lentivirus diffusion and infection did not spread widely from the infusion site; the GFP signal in this image has not been amplified with immunolabeling and tissue was not protected from any fluorescence decay after perfusion, yet there is still clear evidence of eGFP expression. Western blot for GFP confirms strong expression of lentivirus-delivered eGFP as early as 2 weeks post-infusion (Fig. 11.2D).

In Exp.1, NAC tissue was collected from one rat per lentivirus group at 2, 4, 6, 8, and 10 weeks after vector infusion and assayed for D3 expression by Western blot, using three different DRD3 antibodies (Abcam 40655, Abcam 42114, Santa Cruz 9114). No difference in DRD3 expression was observed between groups at Week 2. At Week 4, there appeared to be a decrease in DRD3 expression in Lenti-D3-shRNA rats compared to the Control (Table 11.1, Fig. 11.3) with the Abcam 42114 antibody. However, this effect was not seen with the other two antibodies, and was not replicated in the tissue collected in weeks 6-10 as measured with any antibody.

All DRD3 antibodies were tested for specificity by assaying different rat brain regions, including the dorsal striatum, where there is lower endogenous DRD3

expression, and in the lateral cerebellum, where there is no known dopamine receptor expression. All brain regions demonstrated positive staining with all three antibodies. Additionally, densitometry failed to reveal any consistent regional variation in the strength of staining (Fig. 11.4). This runs contrary to known dopamine receptor distribution, indicating that the commercially available antibodies displayed nonspecific binding in our hands, and Western blot was deemed to be a suboptimal means for assessing D3 expression.

Lentivirus infusion was repeated with the same experimental design in Exp. 2, but tissue was assayed for D3 mRNA expression via quantitative rt-PCR. Tissue collected at Weeks 2 and 4 did not demonstrate any suppression of DRD3 expression, but at Week 6, there was a modest decrease of ~40% in the Lenti-D3-shRNA02 and Lenti-D3-shRNA03 rats (Table 11.2).

Initially, different D3-shRNA constructs were delivered into separate groups of rats in an effort to test for non-specific molecular or behavioral effects (i.e., effects that are present with one construct but not the others). After this method proved to be suboptimal for reducing D3 expression, another experiment was performed in which the viral titer was increased 10-fold and all three D3-shRNA constructs were combined into a "cocktail", as was done in a published report (Bahi et al., 2005). Group sizes were also increased such that 6 animals from either the Control or Active Lenti-D3-shRNA groups were collected at each time point. At weeks 2 and 4, no suppression of D3 expression was observed by quantitative rt-PCR (Fig. 11.5). In the absence of any markers, such as GFP on the D3-shRNA constructs that would signify successful infection of the neurons, and

with no reliable or specific antibodies to DRD3 available for Western blot or immunohistochemistry, no other means for assessing the magnitude or extent of D3 suppression were attempted.

Figure 11.2: Confirmation of successful lentivirus infection within the NAcC. [A]: Light microscopy photo of left, ventral brain. AC is indicated in yellow for reference. [B]: Fluorescence microscopy of the same field shown in B with FITC filter, showing neurons and glia expressing GFP. [C]: Fluorescence microscopy of the same fields shown in B and C with TRITC filter to look for auto-fluorescent artifacts. [D]: Western blot for GFP and β-actin in NAC tissue punches showing expression of CMV-eGFP delivered via lentiviral vector. Blot of GFP standard is shown for comparison in the left-most lane. Tissue was collected 2 weeks post-injection.

Table 11.1: Densitometry of Western blots for FRD3 expression in the rat NAc 4 weeks after lentivirus injection from Exp. 1.

Lentivirus Contruct	DRD3/β-actin Densitometry	Relative to Control
CMV-eGFP	0.660	100%
D3-shRNA-01	0.331	50.1%
D3-shRNA-02	0.233	35.3%
D3-shRNA-03	0.088	13.3 %

Figure 11.3: Western blot for DRD3 and β-actin corresponding to Table 11.1. Active D3-shRNA rats showed decreased DRD3 expression compared to the Control rat in NAc tissue.

Figure 11.4: Western blot of various brain regions with three different DRD3 antibodies. There was little to no regional variation in DRD3 staining, suggesting that antibodies were exhibiting nonspecific binding.

Table 11.2: Quantitative rt-PCR results for DRD3 expression in rat NAC 6 weeks after lentivirus injection from Exp. 2.

Lentivirus Construct	Normalized Values		rDRD3	
	Normalized ng		Fold Increase	
	Average	StDev	Average	StDev
CMV-eGFP	25.84	0.40	1.00	0.02
D3-shRNA-01	25.59	2.11	0.99	0.08
D3-shRNA-02	14.55	0.05	0.56	0.00
D3-shRNA-03	15.38	0.67	0.60	0.03

Figure 11.5: Quantitative rt-PCR of DRD3 expression from Exp. 3. Data are expressed as % of Control levels. No reduction in DRD3 was detected. $N = 6$ rats/group/time point.
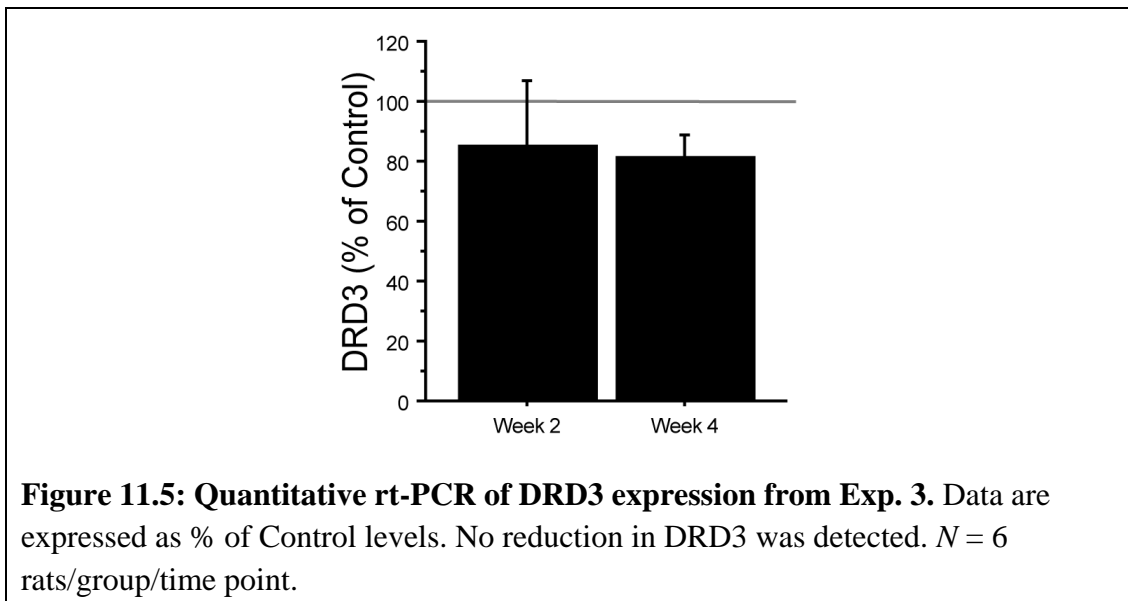
2. Behavioral results: Acoustic startle and PPI were measured before and after lentivirus infusion. The results of Exps. 1 and 2 are shown in Fig. 11.6. In all animals, PPI and startle magnitude were increased on the second test day. There was no significant difference in PPI or startle magnitude between animals injected with different lentivirus constructs.

Behavioral data for the specific groups that showed evidence of decreased DRD3 expression are seen in Fig. 11.7. There were no significant effects of pre- vs. post-injection testing on PPI or startle magnitude. Statistically, behavioral patterns observed after construct infusion did not differ from pre-infusion patterns, as demonstrated by the lack of any significant effects of lentivirus construct or significant construct $\times$ pre/post infusion interactions (all comparisons NS).

With no molecular evidence of D3 suppression, a behavioral signal was probed by testing the 17 remaining rats in Exp. 3 with the D3 preferential agonist pramipexole (PPX) during weeks 6 and 7 after lentivirus infusion. No differences in behavioral effects of the D3 agonist were seen across groups: PPX lowered PPI equally in both Control and Lenti-D3-shRNA infused rats (main effect of PPX dose: $F = 56.24$, df 1,15, $p<0.0001$; no significant interaction of PPX dose $\times$ Lenti group: $F<1$) (Fig. 11.9).

Figure 11.6: Results of behavioral testing before and after lentivirus injection. [Left]: %PPI before and after intensities. There was a significant increase in PPI from pre- to post-injection testing, but no significant effects of lentivirus construct or construct $\times$ pre/post interactions. [Right]: Startle magnitude to P-ALONE trials during PPI testing blocks. Again, there was a significant effect of pre- vs. post-injection testing, but no significant effects of lentivirus, and no significant lentivirus $\times$ pre/post interaction.
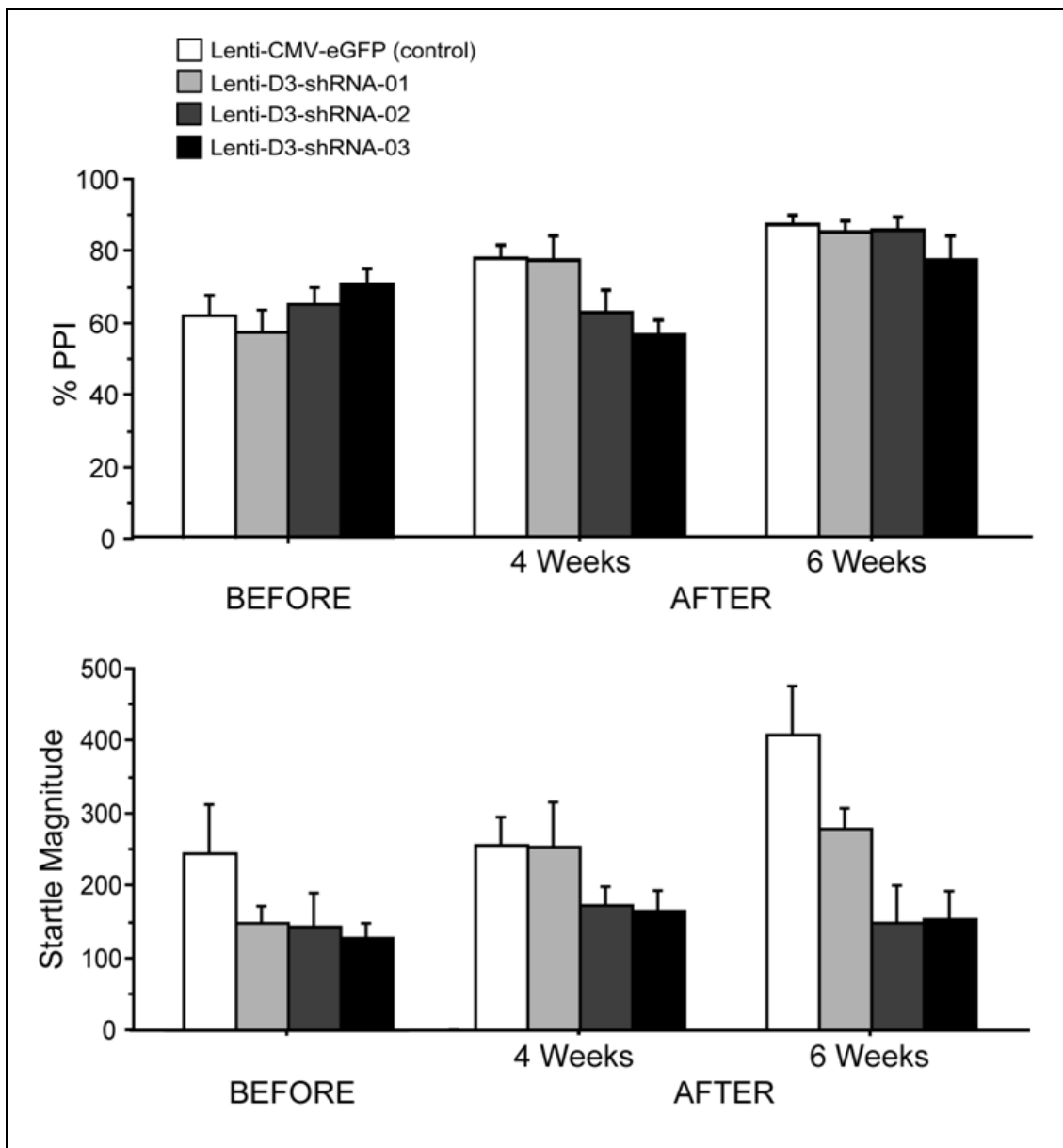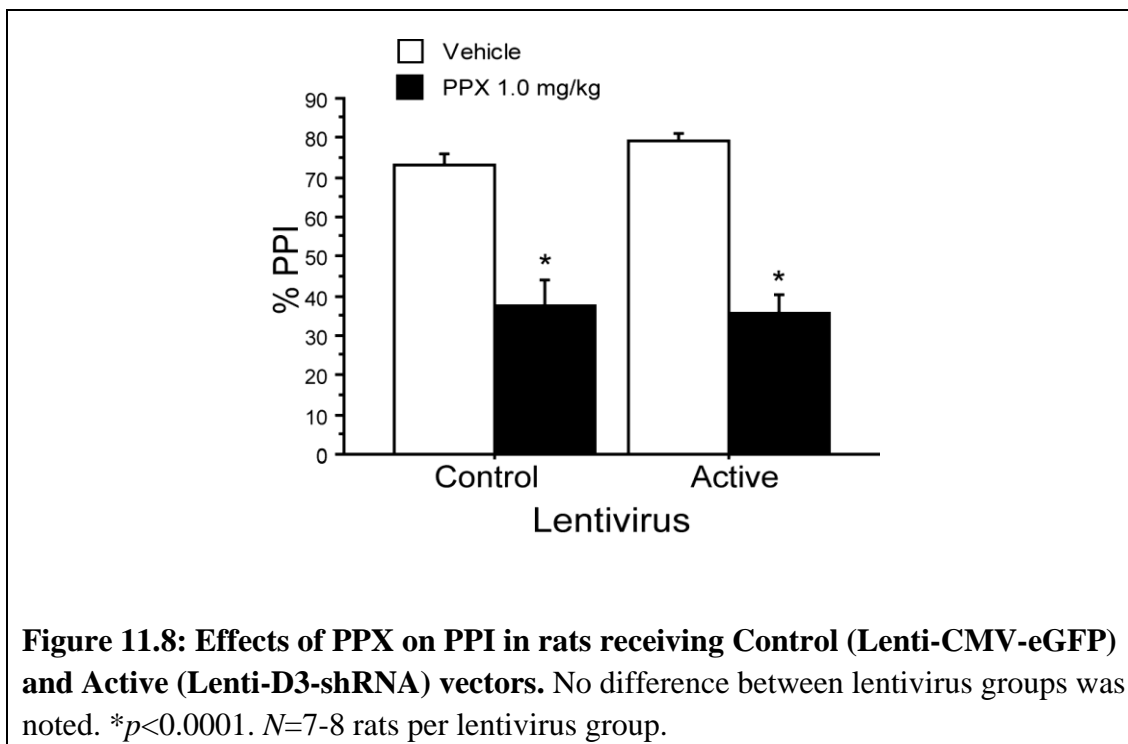
Figure 11.7: Behavioral testing before and 4 and 6 weeks after lentivirus injection. Specific analyses shown here correspond to molecular data shown in Tables 11.1 and 11.2. [A]: %PPI before and after lentivirus injection. There was an expected significant effect of prepulse intensity ($p < 0.0001$), but no significant intensity $\times$ lentivirus or intensity $\times$ pre/post interaction, so data are collapsed across prepulse intensities. There were no significant effects of pre- vs. post-injection testing, as demonstrated by the lack of any significant effects of lentivirus construct or construct $\times$ pre/post interactions. [C]: Startle magnitude to P-ALONE trials during PPI testing blocks. There was a significant effect of pre- vs. post-injection testing and no significant lentivirus $\times$ pre/post interaction.

Figure 11.8: Effects of PPX on PPI in rats receiving Control (Lenti-CMV-eGFP) and Active (Lenti-D3-shRNA) vectors. No difference between lentivirus groups was noted. *$p<0.0001$. N=7-8 rats per lentivirus group.

E. Discussion:

In an attempt to reduce regional levels of D3 receptor expression, shRNA constructs were used that had previously been shown to suppress D3R expression in the adult rat brain (Bahi et al., 2005). Because these constructs did not include a GFP tag, it was impossible to confirm that the lentiviral vectors successfully infected the injected regions, and that cells in those regions subsequently expressed the genetic material delivered by the vectors. The Lenti-CMV-eGFP control condition was used as a proxy, enabling us to detect successful infection and consequent expression of GFP in these animals. Without direct evidence for viral infection from the active lentivirus groups,

however, it was difficult to identify the basis for the apparent failure of receptor knockdown.

It is possible that the viral titer in this study was not high enough. However, the titer used for these studies was well above the 10^7 IU/mL typically suggested to achieve lentiviral vector knockdown (Lasek and Azouaou, 2010). Another possible cause of non-replication of D3 suppression with these shRNA constructs is that the effects are too subtle to be detected in endogenous D3 expression; the previously published report using these constructs used rats that had cocaine-induced overexpression of D3 receptors.

The issue is further complicated by a lack of reliable and specific D3 receptor antibodies. A good antibody would allow for the assessment of D3R expression with higher resolution, using immunohistochemistry and microscopy of tissue. Indeed, the unreliability of commercially available DA receptor antibodies has been reported by others (e.g. Bodei et al., 2009). Autoradiography might have been an alternative strategy for quantifying D3 receptors, though it provides less spatial resolution than does immunohistochemistry, and without evidence of any D3 suppression, the investment required to attempt this approach to D3 labeling was not warranted. Moreover, many of the radioligands that have been used in previous reports of D3 receptor distribution also exhibit nonspecific labeling (Bancroft et al., 1998; Diaz et al., 1995; Herroelen et al., 1994; Rabiner et al., 2009).

These studies do underscore the importance of converging lines of evidence in the interpretation of receptor and behavioral changes after regional shRNA infusion. For example, findings from Wester blot analyses (Table 11.1) initially suggested the

successful suppression of D3 expression at week 4; furthermore, the pattern of suppression seen on Western blot (construct 3 > 2 > 1) appeared to be correspond with the pattern of reduced PPI in these rats during week 4, which (especially compared to pre-injection levels) followed the same pattern (construct 3 > 2 > 1) (Figure 11.5, top, center). However, in checking antibody specificity, comparable levels of positive staining were detected across a number of brain regions, including ones known to have little or no D3 expression. Thus, this control study suggested that the apparent WB evidence of reduced D3 expression was based on the activity of antibodies that lacked D3 specificity; as a result, the WB findings could not be viewed as support for a loss of D3 receptors, and the contemporaneous reduction in PPI could not be attributed to changes in D3 function.

In summary, a reduction in NAC D3 expression could not be convincingly demonstrated after the intracerebral infusion of 3 Lenti-D3-shRNA constructs - either individually or as a high titer "cocktail." A number of control experiments and alternate experimental strategies were employed to understand the basis for this failed D3 suppression, and optimize the chances of successful D3 "knockdown." In its current state, this technique does not appear to be viable for the desired goal of explicating the D3 substrates of behavior and drug sensitivity in rats.

F. Acknowledgements:

CHAPTER 12:

Signaling pathways of the D3 agonist pramipexole in the rat ventral forebrain

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Hammer, Jr., Neal R. Swerdlow

A. Abstract:

1. Background: The dopamine D3 receptor may be a target for development of novel pharmacotherapeutics for several neuropsychiatric disorders, due to its distribution in limbic and mesolimbic regions. Relatively little is known about the *in vivo* signaling pathways downstream of D3 receptor activation due to the substantial homology between D2 and D3 receptors. Cell culture studies with isolated receptor subtype expression have yielded conflicting results regarding the similarity between D2- and D3-activated signaling cascades. D2 receptors are thought to be $G_{i/o}$ protein-linked and primarily act through inhibition of adenylate cyclase; the degree to which D3 receptors act through this pathway is controversial. We have investigated the behavioral profile of D3 vs. D2 activation in rodent measures relevant to antipsychotic development, such as prepulse inhibition of acoustic startle and locomotor activity. Here, we analyzed changes in signaling cascade elements induced by D3- or D2-preferential agonists within brain regions that regulate the behavioral effects of these drugs.

2. Methods: Pramipexole (PPX) and sumanirole (SUM) were used as D3- and D2-preferential agonists, respectively; the D1/D2-family agonist apomorphine (APO) and

the D2/D3 agonist ropinirole were also used as comparison drugs for some studies. DA-related signal transduction changes were studied within brain regions that included the nucleus accumbens (core and shell, NAcC and NAcS) and medial caudatoputamen (mCPu) of Sprague-Dawley rats. $G_{i/o}$ protein activation was measured in fresh-frozen brain slices by autoradiography of $[^{35}S]GTP\gamma S$ binding in the presence of PPX. PKA activity was measured by ELISA, and phosphorylation of CREB and ERK1/2 as well as c-Fos expression were measured by immunohistochemistry. Brain tissue was collected in conditions that mimicked behavioral testing of DA agonists. D3 vs. D2 contributions to agonist effects were tested with the D3- and D2-preferential antagonists U99194 and L741626, respectively.

3. Results: PPX significantly increased $[^{35}S]GTP\gamma S$ binding in the NAcC, NAcS, and mCPu, suggesting PPX-induced $G_{i/o}$ protein activation. Both PPX and SUM reduced CREB and ERK phosphorylation in these same regions, while only PPX significantly reduced c-Fos expression. This c-Fos signal was opposed within the NAC by the D3-preferential antagonist, U99194, while the D2-preferential antagonist L741626 failed to oppose the PPX effects and independently activated c-Fos expression.

4. Conclusions: With the most selective agonists commercially available, we identified elements of the intracellular signaling system that respond to D2 and D3 receptor activation. Namely, *in vivo* and *ex vivo* administration of PPX activated $G_{i/o}$ proteins and inhibited CREB and ERK1/2, similarly to D2 and non-selective D2/D3 agonists. Interestingly, the present results demonstrate a PPX-induced suppression of NAc c-Fos expression, which was not observed after SUM administration and was opposed by D3- but not D2-blockade. This divergence of D3 vs. D2 effects on NAc

intracellular signaling might reflect distinct receptor-specific signal pathways that could potentially be exploited for the development of novel pharmacotherapies.

B. Introduction:

Within the family of dopamine (DA) D2-like receptors, the D3 subtype is a potential target for more specific pharmacotherapeutics with a more favorable side effect profile than those currently available. Due to the structural similarities between the D2 and D3 receptors (Livingstone et al., 1992; Sokoloff et al., 1990), it has been difficult to preferentially target the D3 receptor *in vivo* to study its role in animal models relevant to preclinical drug development and characterize the intracellular pathways that mediate these behavioral effects. *In vitro* studies have yielded conflicting findings regarding the similarities and differences between D2 and D3 receptor signaling pathways (cf. Ahlgren-Beckendorf and Levant, 2004). As more selective tools become commercially available, though, these questions can begin to be answered.

One animal model of sensorimotor gating that is widely used in the development of antipsychotic medication is prepulse inhibition (PPI) of acoustic startle. The neural circuitry regulating PPI in rats has been elucidated (cf. Swerdlow et al., 2000, 2001a) and includes regulation by forebrain DA activity mediated by D2-family receptor activation, particularly in the nucleus accumbens (NAc), and by the subsequent reduction in GABA release in the ventral pallidum and perhaps other NAc projection. Currently, the intracellular events between D2-family receptor activation and downstream reduction in GABA release are not well understood, though some key signaling molecules are being

confirmed with *in vivo* methods: the nonspecific D1/D2-family agonist apomorphine (APO) has been shown to reliably disrupt PPI in rats (Saint Marie et al., 2006; Swerdlow et al., 2011) at doses and time frames that correspond to decreases in CREB phosphorylation and c-Fos expression in the NAc.

In the present studies, we have examined changes in these and other signaling elements after D2 or D3 receptor activation under conditions that are similar to those producing DA agonist-induced PPI deficits. We have extensively characterized the behavioral effects of the D3-preferential agonist pramipexole (PPX) (Chang et al., 2010b, 2011; Swerdlow et al., 2009a; Weber et al., 2008a, 2009b), and have shown that PPX may cause disruption in PPI through mechanisms that are relatively insensitive to blockade by the D2-preferential antagonist L741626, and that this effect is separable from behavioral effects of PPX on startle magnitude and locomotor activity. We now seek to identify commonalities and points of divergence in the intracellular signaling pathways of D2 and D3 receptors in forebrain regions regulating PPI. Here, we report changes in [^{35}S]GTPγS binding, CREB phosphorylation, ERK1/2 phosphorylation, and c-Fos expression after DA receptor stimulation with APO, PPX, and the D2-preferential agonist sumanirole (SUM).

C. Methods

1. Animals: Adult male Sprague-Dawley rats (225-250 g; Harlan Laboratories, Livermore, CA) were housed 2-3 animals per cage and maintained on a reversed light/dark schedule with food and water available *ad libitum*. All testing or tissue

collection occurred during the dark phase, and rats were handled within 2 d of arrival and allowed to acclimate for at least 7 days before and procedures.

2. Drugs: All drugs were administered sc. in a volume of 1 ml/kg body weight. For specific information, see Table 12.1.

Table 12.1: Drug information

Drug	Vendor	Class	Vehicle	Dose (mg/kg)
L741626	Tocris	Antagonist	0.1% Lactic Acid in Sterile Water	0, 0.03
U99194	Tocris	Antagonist	Saline	0, 10.0
Sumanirole (SUM)	NIMH	Agonist	Saline	0, 3.0
Apomorphine	Sigma	Agonist	0.01% Ascorbic Acid in Sterile Water	0, 0.25
Pramipexole (PPX)	TRC	Agonist	saline	0, 0.3, 1.0

3. [^{35}S]GTPγS binding: Animals naïve to drug were decapitated and their brains were rapidly frozen in 2-methylbutane at -35°C, then stored at -80°C until sectioning using a -20°C cryostat at 20 μm. Serial sections were collected between levels corresponding to 2.2-1.2 mm anterior to bregma (Paxinos and Watson, 1997) (Fig. 12.1A) and thaw-mounted onto SuperFrost Plus slides (2-4 sections per slide). Slides were stored at -80°C prior to processing.

[^{35}S]GTPγS binding was conducted as described previously (Culm et al., 2003; buffer (50 mM Tris-HCl, 2 mM MgCl, 0.2 mM EGTA, 100 mM NaCl, and 0.2 mM DTT pH 7.4) for 15 min at 25°C followed by a 15 min incubation in the same buffer with the

addition of 2 mM GDP (ICN; Costa Mesa, CA). Sections were then incubated in assay

buffer containing 2 mM GDP and 50 pM [^{35}S]GTPγS (NEN-Perkin-Emer Life Sciences,

Boston, MA) in the absence (basal) or presence of DA (100 μM), PPX (10, 100, or 1000

μM), ropinirole (10, 100 or 1000 μM) or apomorphine (1000 μM) (TRC, North York,

Ontario, Canada) for 1h at 25˚C. Only a small number of sections were available for

incubation with apomorphine. A low Mg$^+$ concentration is used to favor labeling of G$_i$

proteins because G$_s$ activation requires much higher Mg$^+$ concentration (25-50 mM;

Waeber and Moskowitz, 1997). After incubation, sections were washed three times in

ice-cold 50 mM Tris-HCl (pH 7.4) and once in ice-cold distilled water. Slides were

allowed to dry for two hours and then exposed to X-ray film (Biomax MR, Eastman

Kodak Company, Rochester, NY) for 12 hours. Relative [^{35}S]GTPγS binding was

determined using a calibration curve based on ^{14}C radiostandards (ARC-146; American

Radiolabeled Chemicals St. Louse, MO) which were co-exposed on the film.

4. [^{35}S]GTPγS binding image analysis: Quantitative autoradiographic analysis of

[^{35}S]GTPγS binding was conducted in the NAc core (NAcC) and shell (NAcS) and

medial caudoputamen (mCPu) bilaterally while blind to binding condition (Fig. 12.1B).

Autoradiographic images were analyzed using NIH ImageJ (developed by Wayne

Rasband, NIMH; available on the Internet at http://rsb.info.nih.gov/nih-image/). Mean

data were calculated for all conditions in each region in all animals.

5. PKA Activity ELISA: 20 min after drug administration, rats were decapitated and brains rapidly dissected and chilled in ice-cold saline. NAC samples were obtained with a 2 mm diameter punch from 1 mm thick coronal slabs (1.2-2.2 mm anterior to Bregma)(Fig. 12.1C) and flash frozen. Tissue was homogenized in extraction buffer and centrifuged. Supernatant was collected and protein concentration measured before PKA activity was measured by ELISA (Assay Designs PKA Kinase Activity Kit (Non-Radioactive)). Samples were loaded in triplicate, with and without PKA inhibitor (PKI), and data are expressed as the ratio between optical density (OD) at 450 nm of sample wells and sample+PKI wells.

6. Immunohistochemistry: All immunohistochemistry procedures are similar to those reported previously (Saint Marie et al., 2006; Swerdlow et al., 2011). Rats were acclimated in the laboratory in individual cages for 2+h and then injected with vehicle or agonist in pCREB, pERK, or c-Fos experiments. For antagonist studies, the first injection of antagonist occurred after the 2h acclimation. See Table 12.2 for timing of perfusion.

Table 12.2. Timeline of perfusion procedures for pCREB, pERK, and c-Fos immunohistochemistry

Relative Time (HH:MM)	Event	
	pCREB/pERK	c-Fos*
0:00	Agonist injection Into chamber with background noise	Agonist injection Into chambers with no noise
0:20	Anesthesia	Back into individual cage
0:25	Perfusion	
1:55		Anesthesia
2:00		Perfusion

*for studies involving antagonist pretreatment, animals received either U99194 (0, 10.0 mg/kg) 1 hour prior or L741626 (0, 0.3 mg/kg) 30 min prior to agonist injection.

Startle chambers had 70 dB(A) background broad band noise for pCREB/pERK studies to simulate behavioral testing of prepulse inhibition from other studies (Chang et al., 2010b; Swerdlow et al., 2009a; Weber et al., 2008a, 2009b, 2010b). Rats were anesthetized with a lethal overdose of pentobarbital and perfused under deep anesthesia.

The perfusion consisted of a brief washout with phosphate-buffered (pH 7.4) saline. pCREB studies had studies used a 1 min, room-temperature washout. Animals were then perfused for 2.5 min with ice-cold or room temperature phosphate-buffered 4% paraformaldehyde (±50 mM NaF and ±5 mM sodium orthovanadate). Brains were dissected out and chilled at 4°C in the final perfusate overnight and then transferred to a cryoprotective solution of 30% sucrose in perfusate for 3 days at 4°C. Next, brains were frozen-sectioned in the transverse plane at 40 μm and collected in Tris-buffered (pH 7.5)
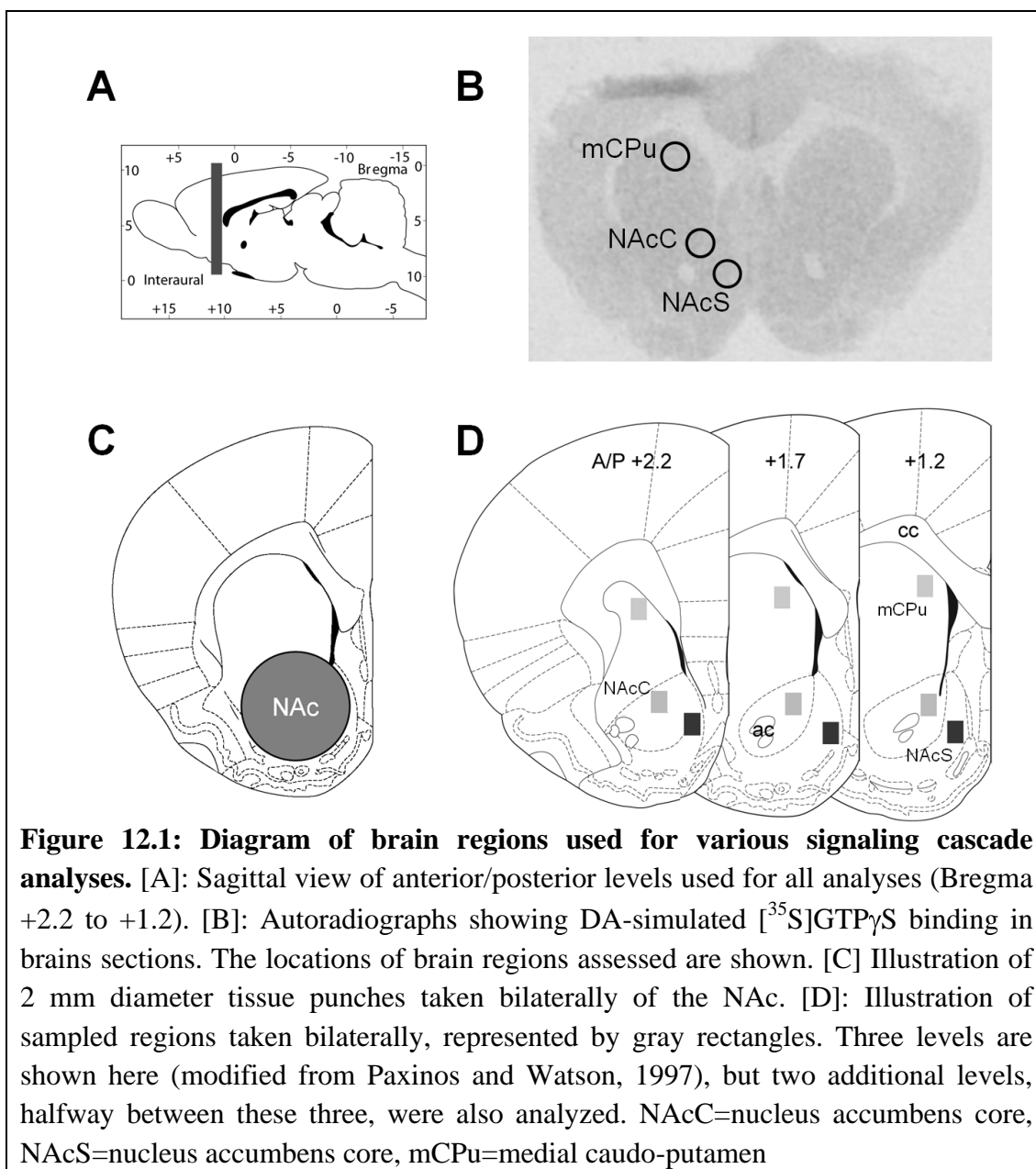
saline (TBS) ($\pm$50 mM NaF and $\pm$5 mM sodium orthovanadate). Sections at ~160 μm intervals (paired adjacent sections for pCREB and CREB) from levels corresponding to 2.2-1.2 mm anterior to bregma (Paxinos and Watson, 1997) were rinsed, floating, in TBS and then 0.5% H_2O_2 in TBS to quench endogenous peroxidase activity, rinsed in TBS again, and incubated for 2 h in blocking solution containing 10% normal horse serum and 0.33% Triton X-100 in TBS ($\pm$ anti-phosphatases). Section were next incubated overnight with rabbit antibody to: pCREB diluted 1:300 (phosphorylated at serine 133, Cell Signaling Technology; Danvers MA, USA), CREB diluted 1:2000 (Cell Signaling Technology), pERK diluted 1:1200 (Cell Signaling Technology), or c-Fos diluted 1:5000 (Calbiochem). Sections were rinsed and incubated for 4 h in biotinylated anti-rabbit IgG secondary antibody (Jackson ImmunoResearch; West Grove, PA, USA) and visualized via Vectastain Elite ABC kit with a Ni-enhanced DAB reaction (Vector Laboratories, Burlingame, CA, USA).

7. Immunohistochemistry image analysis: Regions of the NAcC, NAcS, and mCPu were sampled at 5 AP levels (Fig. 12.1D) and digitized with a Leitz Laborlux microscope, ×20 objective, and Polaroid DMC1 digital camera. Illumination was set to maximize gray level range without saturation, and an image from a blank slide was taken at the beginning of each collection session to correct images to be quantified for non-uniformities in background illumination. Images (1,200 × 1,600 pixels, equivalent to 380 × 505 μm of tissue section) were collected while blind to experimental conditions, and all subsequent processing was automated to avoid investigator bias. Studies of the

cytoplasmic protein pERK quanitifed average gray level for the entire image field. For studies of pCREB, CREB, and c-Fos, local contrast was enhanced with a batch file in Adobe PhotoShop using an unsharp mask (amount, 250%; threshold, 64 gray levels; radius, 25 pixels), and positively labeled nuclei were identified and counted with a macro-file constructed in ImagePro Plus (ver. 4.0, Media Cybernetics; Bethesda, MD, USA) that automatically thresholded objects based on gray level (0-127) and size (area $\geq$100 pixels). For pCREB and CREB, labeling was expressed as the total area occupied by objects in the captured field that met criteria rather than counting individually stained nuclei because of the large numbers of positively-stained nuclei in 40 μm thick sections. CREB phosphorylation is expressed as a percent area of the image field occupied by pCREB-stained nuclei compared to the area occupied by CREB-stained nuclei from an immediately adjacent tissue section. For c-Fos studies, staining is expressed as the number of positively-stained nuclei that met criteria.

8: Statistical Analysis: Autoradiography of [^{35}S]GTPγS binding, PKA activity, and pCREB, pERK, or c-Fos immunostaining all yielded quantitative values for each animal/condition/sample, which were analyzed by ANOVA. Where a large degree of variability was present, data were analyzed with an equality of variances F tests, and if appropriate ($p<0.05$), with a Kruskal Wallis test and separate Mann Whitney tests for each drug group compared to vehicle. One vehicle-group rat exhibited c-Fos levels across all regions that were >> 3 SD above the group mean and were excluded from analysis on this basis. Non-parametric tests were used for the PKA and pCREB/CREB data sets. For

all other studies, post-hoc comparisons were conducted using Fisher's PLSD. Unless otherwise noted, data are averaged bilaterally and, for studies involving serial brain sections, collapsed across A/P level. Of the four sections mounted on each slide for [^{35}S]GTPγS binding studies, a small number were unusable on one or both sides because of folded or torn tissue, so average values of all section on each slide were calculated.

Figure 12.1: Diagram of brain regions used for various signaling cascade analyses. [A]: Sagittal view of anterior/posterior levels used for all analyses (Bregma +2.2 to +1.2). [B]: Autoradiographs showing DA-simulated [^{35}S]GTPγS binding in brains sections. The locations of brain regions assessed are shown. [C] Illustration of 2 mm diameter tissue punches taken bilaterally of the NAc. [D]: Illustration of sampled regions taken bilaterally, represented by gray rectangles. Three levels are shown here (modified from Paxinos and Watson, 1997), but two additional levels, halfway between these three, were also analyzed. NAcC=nucleus accumbens core, NAcS=nucleus accumbens core, mCPu=medial caudo-putamen
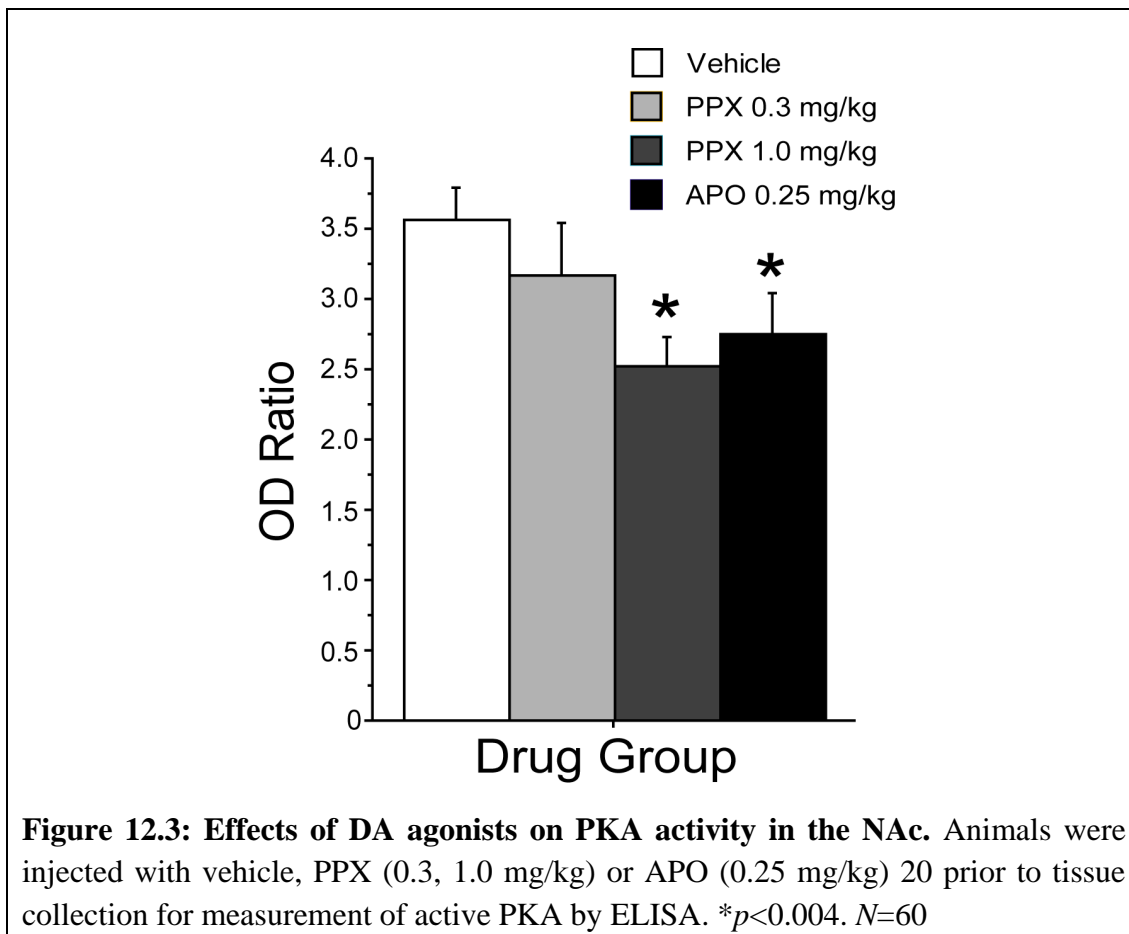
D. Results:

1. [^{35}S]GTPγS binding: In general, DA and DA agonists stimulated [^{35}S]GTPγS binding across most brain regions. ANOVA of binding, with drug concentration as a between-subject factor and brain region as a within-subject factor revealed significant main effects of DA (F=5.50, df 1,20, p<0.03) and PPX (F=27.00, df 3,30, p<0.0001). Ropinirole exhibited inverted-U concentration effects, with significant stimulatory effects of 100 μM in the NAcC and mCPu (p's < 0.05). Only a small number of sections were available for assessing APO effects, which still achieved significance within the NAcC and mCPu (p's < 0.05). Of most relevance to these studies, [^{35}S]GTPγS binding was significantly increased by PPX in the NAcC (F=16.83, *df* 3,30, *p*<0.0001), NAcS (F=20.64, *df* 3,30, *p*<0.0001), and mCPu (F=44.70, *df* 3,30, *p*<0.0001) (Figure 12.2). Post-hoc tests showed that [^{35}S]GTPγS binding accompanying the highest concentration of PPX was significantly different from basal conditions in all three regions (*p*<0.0001), and in the mCPu, 100 μM of PPX also significantly increased binding (*p*<0.02).

Figure 12.2: Effects of PPX on [³⁵S]GTPγS binding in different brain regions. Within the NAcC, NAcS, and mCPu, there was a significant increase in binding with the highest concentration of PPX. *$p<0.02$, **$p<0.0001$. DA=dopamine, PPX=pramipexole, APO=apomorphine, ROP=ropinirole, N=7-11 slides/condition.
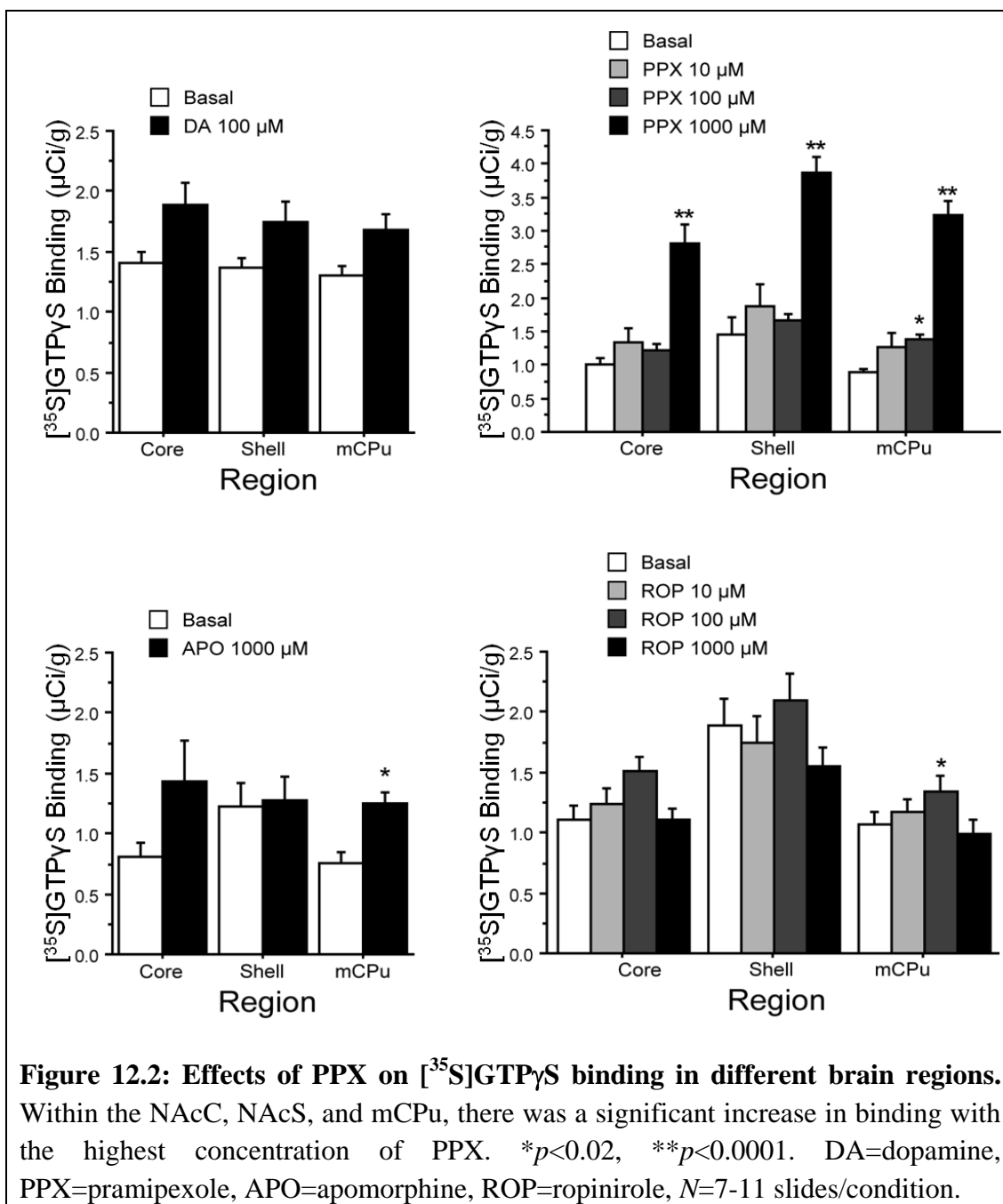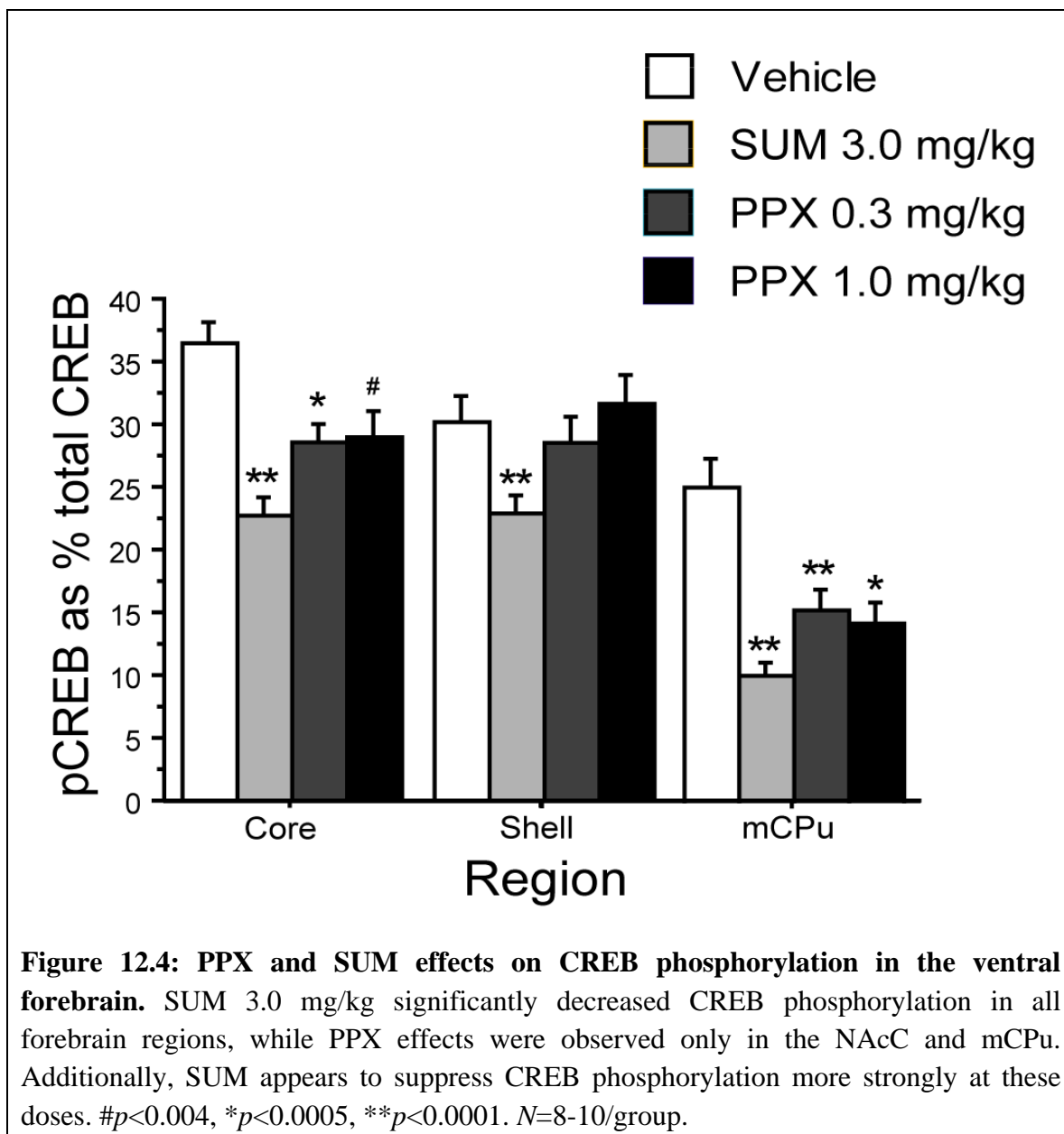
2. PKA Activity: Equality of variances F test showed significant differences in variance between groups ($p<0.05$), so nonparametric tests were used in subsequent analyses. Kruskal Wallis test revealed a significant effect of drug ($p<0.0001$). Separate Mann-Whitney tests comparing vehicle and each drug group revealed a trend towards

reduced PKA activity by PPX 0.3 mg/kg ($p=0.06$), and significant reductions on PKA activity in the PPX 1.0 mg/kg and APO 0.25 mg/kg groups ($p's<0.004$)(Fig. 12.3).
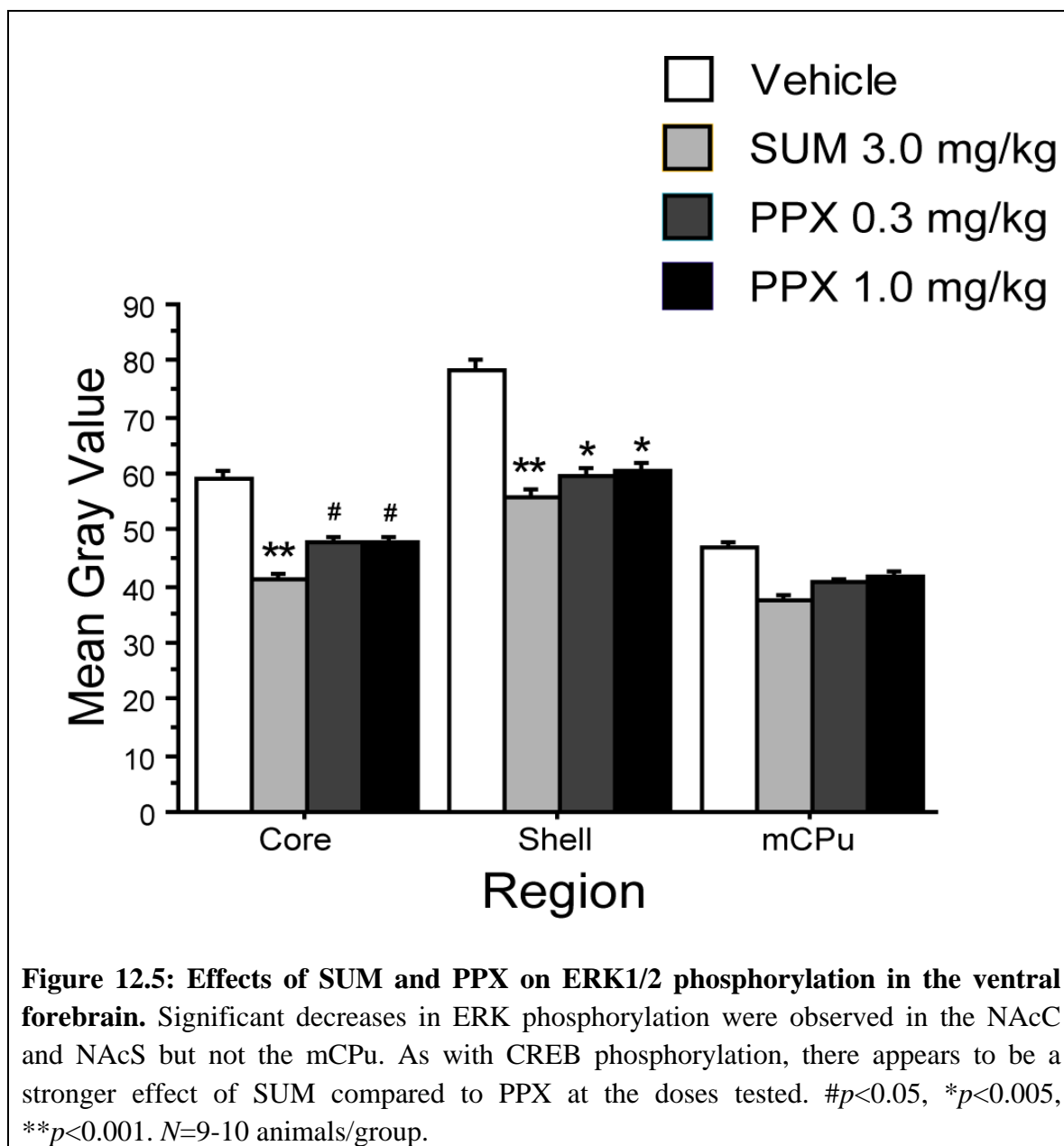

Figure 12.3: Effects of DA agonists on PKA activity in the NAc. Animals were injected with vehicle, PPX (0.3, 1.0 mg/kg) or APO (0.25 mg/kg) 20 prior to tissue collection for measurement of active PKA by ELISA. *$p<0.004$. $N=60$

3. CREB phosphorylation: CREB phosphorylation as a percent of total CREB was found to have a high level of variability, with significant differences identified via an equality of variances F test ($p<0.05$). Kruskall Wallis tests found significant effects of drug group in the NAcC ($p<0.0001$), NAcS ($p<0.0008$), and mCPu ($p<0.0001$). Post-hoc Mann-Whitney tests showed that, compared to the vehicle group, there was significantly decreased CREB phosphorylation with SUM in all three forebrain regions ($p's<0.0001$). PPX 0.3 mg/kg also significantly reduced CREB phosphorylation in the NAcC

($p<0.0003$) and mCPu ($p<0.0001$), but not the NAcS ($p=0.065$). PPX 1.0 mg/kg significantly reduced CREB phosphorylation in the NAcC ($p<0.004$) and mCPu ($p<0.0004$)(Fig. 12.4).
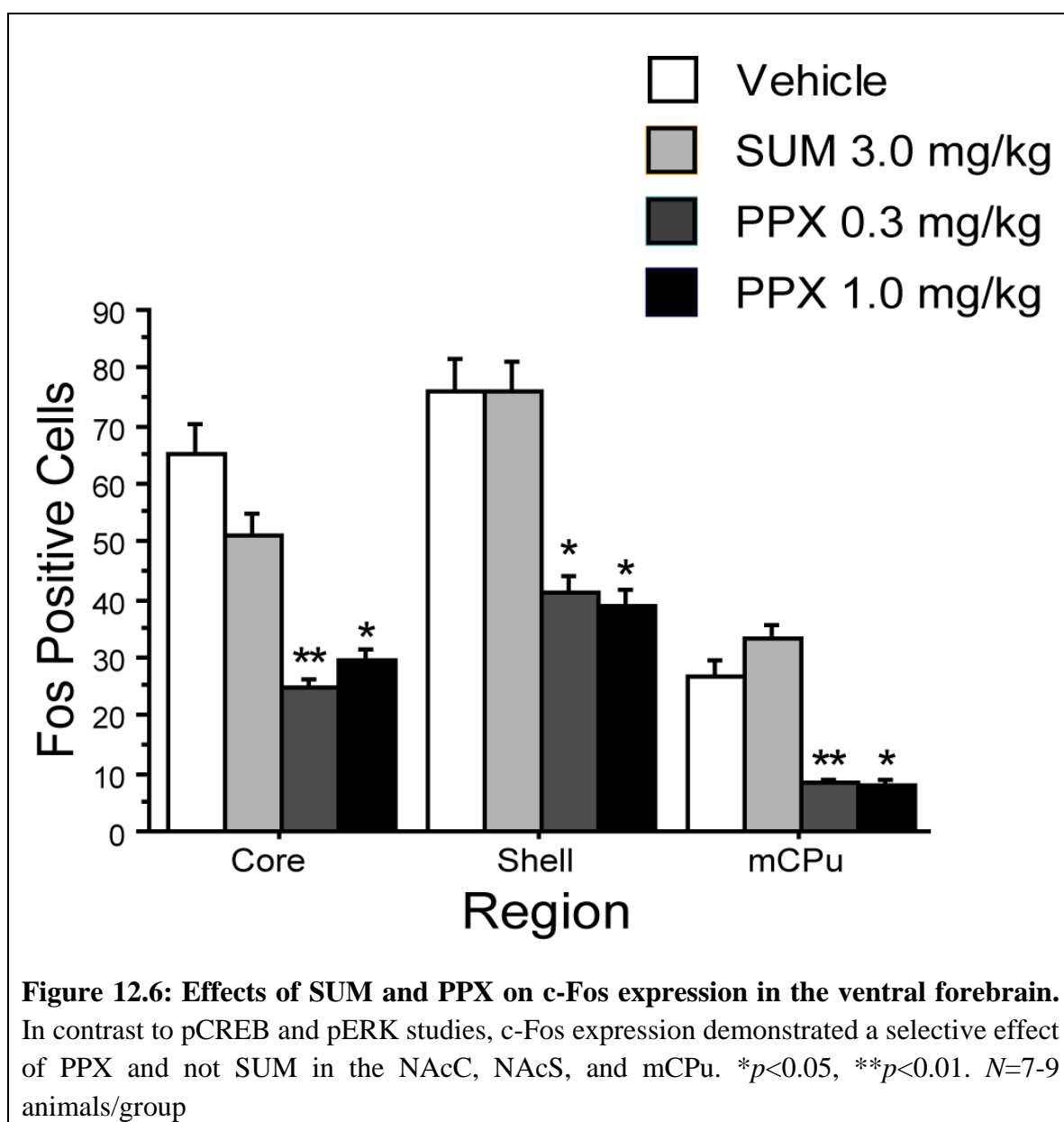

Figure 12.4: PPX and SUM effects on CREB phosphorylation in the ventral forebrain. SUM 3.0 mg/kg significantly decreased CREB phosphorylation in all forebrain regions, while PPX effects were observed only in the NAcC and mCPu. Additionally, SUM appears to suppress CREB phosphorylation more strongly at these doses. #$p<0.004$, *$p<0.0005$, **$p<0.0001$. $N=8$-10/group.

4. ERK1/2 phosphorylation: In the NAcC, RM ANOVAs revealed a significant main effect of drug (F=4.82, *df* 3,34; *p*<0.007). Post-hoc testing indicated that SUM (*p*<0.0007), PPX 0.3 and 1.0 mg/kg (*p's*<0.03) groups all had significantly less pERK staining than the vehicle group. In the NAcS, there was also a main effect of drug (F=6.78, *df* 3,34; *p*<0.002), which post-hoc testing revealed was due to significant decreases of pERK in SUM (*p*<0.0003), PPX 0.3 mg/kg (*p*<0.002), and PPX 1.0 mg/kg (*p*<0.003) conditions compared to vehicle. In contrast, there were no significant changes in ERK phosphorylation in the mCPu, despite a trend towards a main effect of drug (F=2.70, *df* 3,34; *p*=0.061;NS)(Fig. 12.5).

Figure 12.5: Effects of SUM and PPX on ERK1/2 phosphorylation in the ventral forebrain. Significant decreases in ERK phosphorylation were observed in the NAcC and NAcS but not the mCPu. As with CREB phosphorylation, there appears to be a stronger effect of SUM compared to PPX at the doses tested. #$p<0.05$, *$p<0.005$, **$p<0.001$. N=9-10 animals/group.

5. c-Fos expression: RM ANOVAs of c-Fos counts in the NAcC showed significant main effects of drug (F=4.44, df 3,27, $p<0.02$), with significant changes after PPX 0.3 mg/kg ($p<0.004$) and 1.0 mg/kg ($p<0.02$) in post-hoc tests. There was also a significant main effect of drug in the NAcS (F=3.06, df 3,27; $p<0.05$), again due to effects of PPX 0.3 and 1.0 mg/kg ($p's<0.05$) vs. vehicle. In the mCPu, the drug effect was

again significant (F=8.05, *df* 3,27; *p*<0.0006), with significantly decreased c-Fos counts after PPX 0.3 mg/kg (*p*<0.008) and 1.0 mg/kg (*p*<0.02). Significant interactions were also detected for drug × AP level for the NAcC (F=3.75, df 12, 108, p<0.0001), NAcS (F=1.97, df 12,108; p<0.04) and mCPu (F=2.49, df 12,108; p<0.007), in each case reflecting the distributional properties of significant c-Fos-suppressing effects of PPX and not SUM (Fig. 12.6).
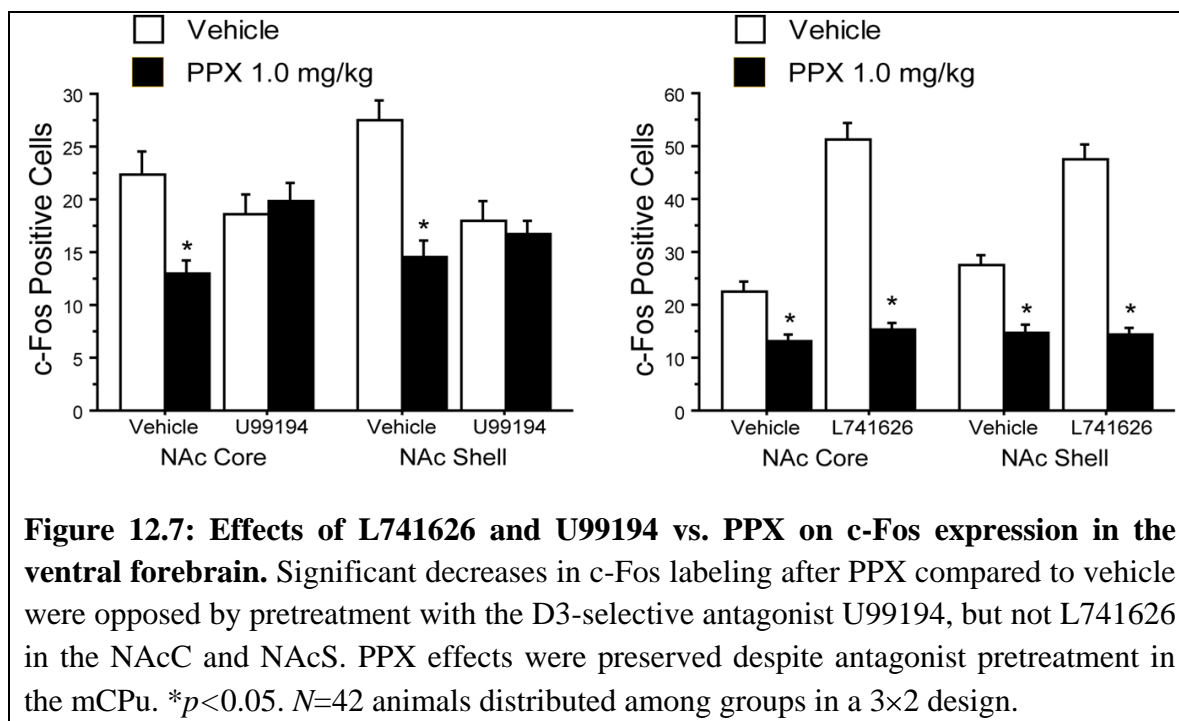

Figure 12.6: Effects of SUM and PPX on c-Fos expression in the ventral forebrain. In contrast to pCREB and pERK studies, c-Fos expression demonstrated a selective effect of PPX and not SUM in the NAcC, NAcS, and mCPu. **p<0.05, **p<0.01. N=7-9* animals/group

6. c-Fos expression with antagonists/agonist combinations: Because c-Fos expression demonstrated the most "D3-selective" profile by showing significant alterations with the D3-preferential agonist PPX but not the D2-preferential agonist SUM, we next assessed the effects of PPX on c-Fos after pretreatment with the D2- and D3-preferential antagonists L741626 and U99194, respectively. Animals received injections of vehicle (divided amongst vehicles for L741626 and U99194), L741626 (1.0 mg/kg), or U99194 (10.0 mg/kg), followed at appropriate intervals by either vehicle or PPX (1.0 mg/kg). Preliminary and published studies determined that this dose of L741626 significantly opposes SUM- but not PPX-disrupted PPI (Weber et al., 2009b, 2010b), and that this dose of U99194 opposes PPX-disrupted PPI (Breier et al., 2011). Analyses confirmed no differential effect of pretreatment vehicles (saline or lactic acid) on c-Fos expression, and thus vehicle groups were combined for subsequent analyses. Separate ANOVAs revealed that the PPX-induced suppression of c-Fos expression in the NAc was opposed by U99194 pretreatment, but not by L741626 pretreatment (Fig. 12.8)

For U99194-pretreated rats, ANOVA of c-Fos expression across both NAc subregions revealed no significant effect of pretreatment ($F<1$), but near-significant main effects of PPX treatment ($F=4.16$, df 1,24, $p<0.053$) and pretreatment $\times$ treatment ($F=4.15$, df 1,24, $p<0.053$) in the predicted directions. Post-hoc analyses showed significant c-Fos-reducing effects of PPX in animals pretreated with vehicle ($p<0.015$) but not among those pretreated with U99194 ($F<1$); among PPX-treated rats, c-Fos expression was arithmetically increased by U99194 pretreatment, although this effect only approached statistical significance in the NAcC ($p = 0.10$).

For L741626-pretreated rats, ANOVA of c-Fos expression across both NAc subregions revealed significant main effects of pretreatment (F=8.98, df 1,24, p<0.007) and PPX treatment (F=29.14, df 1,24, p<0.0001), and a significant interaction of pretreatment × treatment (F=7.62, df 1,24, p=0.01). Interestingly, this interaction reflected significant *c-fos*-activating effects of L741626 pretreatment among rats treated with vehicle (p<0.008). Despite this activation, PPX still significantly suppressed c-Fos expression among L741626-pretreated rats (p<0.002).

In contrast to the NAc, within the mCPu, ANOVA of c-Fos expression after U99194 pretreatment showed no significant main effect of pretreatment (F<1), significant c-Fos-suppressing effects of PPX treatment (F=21.78, *df* 1,24; *p*<0.0001), and no pretreatment × treatment interaction (F<1). Effects of L741626 within the mCPu paralleled those in the NAc: ANOVA confirmed significant main effects of pretreatment (F=8.31, df 1,24, p<0.009) and PPX treatment (F=27.92, df 1,24, p<0.0001), and a significant interaction of pretreatment × treatment (F=6.62, df 1,24, p=0.02). As in the NAc, this interaction reflected significant *c-fos*-activating effects of L741626 pretreatment among rats treated with vehicle (p<0.02), yet, despite this activation, PPX still significantly suppressed c-Fos expression among L741626-pretreated rats (p<0.003)(Fig. 12.7).

Figure 12.7: Effects of L741626 and U99194 vs. PPX on c-Fos expression in the ventral forebrain. Significant decreases in c-Fos labeling after PPX compared to vehicle were opposed by pretreatment with the D3-selective antagonist U99194, but not L741626 in the NAcC and NAcS. PPX effects were preserved despite antagonist pretreatment in the mCPu. *$p<0.05$. *N*=42 animals distributed among groups in a 3×2 design.

E: Discussion:

The present set of studies employed various techniques of autoradiography, ELISA, and immunohistochemistry to measure DA agonist-mediated changes in intracellular signaling - activation of $G_{i/o}$ protein, PKA, CREB, ERK1/2, and expression of c-Fos - in rat brain tissue. By comparing the effects of the D2-preferential agonist SUM, and the D3-preferential agonist PPX in these measures, it was possible to identify points of convergence and divergence among the signaling pathways activated by stimulation of D2 and D3 receptors. The effects of these two drugs on behaviors such as prepulse inhibition of startle have been reported previously (Chang et al., 2010; Swerdlow et al., 2009a; Weber et al., 2008a, 2009b, 2010b), and in most cases, the conditions leading up to collection of brain tissue for the signaling assays simulated those used in these behavioral studies.

Both D2 and D3 receptors are G protein-coupled receptors (GPCRs) that are thought to bind $G_{i/o}$ proteins, in opposition to the D1-like family of receptors, which are coupled to G_s. Thus, studies first examined PPX effects on [^{35}S]GTPγS binding under conditions of low Mg^+ concentration, which favor G_i proteins, and compared them to the effects of DA and other DA agonists. For these studies, brain tissue was directly incubated in a solution containing DA receptor agonists, and DA-stimulated binding was also assessed for comparison. PPX elicited a dose-dependent increase in [^{35}S]GTPγS binding; increased binding was also detected after incubation with DA, the mixed D1/D2 agonist, APO, and the mixed D2/D3 agonist, ropinirole. While the magnitude of [^{35}S]GTPγS binding changes among the different drugs did not correspond to their relative potency on PPI disruption, many experimental factors could account for this discrepancy - such as differences in bioavailability *in vivo* vs. solubility *in vitro*, etc. Furthermore, the process of serial tissue section acquisition for slide-based studies introduced a systematic constraint such that incubation with each drug was inevitably linked to a specific rostral-caudal level in the forebrain; differential drug effects on [^{35}S]GTPγS binding could thus easily reflect anatomical differences in receptor distribution across the anterior-posterior extent of the NAc and mCPu. Given this constraint, the general consistency in the changes in [^{35}S]GTPγS binding in response to DA, PPX, APO and ropinirole is quite notable.

The D2 receptor is known to exert many of its actions through inhibition of adenylate cyclase (DeCamilli et al., 1979; Huff, 1996), thus decreasing activity in downstream cascade elements. Several studies have suggested that the D3 receptor either do not (MacKenzie et al., 1994; McAllister et al., 1993; Sokoloff et al., 1990; Tang et al.,

1994) or do affect adenylate cyclase activity (Chio et al., 1994; Cox et al., 1995; Griffon et al., 1997; Hall and Strange, 1999; Robinson et al., 1997), though perhaps with a more limited range of adenylate cyclase isozymes (Robinson et al., 1997). Here, we assessed PPX effects on two signaling elements downstream of adenylate cyclase: PKA and CREB. Additionally, ERK1/2 activity was measured, as it has also been reported to be modulated by D3Rs (Beom et al., 2004; Bruins Slot et al., 2007; Culm et al., 2004). All three of these signaling molecules showed similar decreases in activity after PPX, as well as APO or SUM.

The observed drug effects on NAc signal elements did not necessarily correspond in any direct manner to their effects on NAc-mediated behavioral measures such as prepulse inhibition of startle (PPI): for example, SUM decreased NAc CREB and ERK1/2 phosphorylation to a greater degree than did PPX, while the behavioral effects of systemically-administered SUM on PPI are weaker than those of PPX (Chang et al., 2010b, Weber et al., 2010b). Furthermore, while its behavioral effects on PPI are clearly dose-dependent (Weber et al., 2008a), the effects of PPX on some intracellular measures (e.g. $[^{35}S]$GTPγS binding) appeared to be dose-dependent, while its effects on others (e.g. CREB and ERK1/2 phosphorylation and c-Fos expression) did not.

Of the signaling cascade elements investigated, only c-Fos showed a differential effect of PPX compared to SUM. Induction of the immediate early gene *c-fos* and expression of its protein product, c-Fos, has been widely used in the brain and elsewhere as a tool for mapping sites of cell activation (Herrera and Robertson, 1996; Hoffman and Lyo, 2002; Kaczmarek and Robertson, 2002), and is regarded as one of the first signs of a genomic response to a stimulus (Sheng and Greenberg, 1990). Induction of *c-fos*

expression is part of a preclinical profile suggestive of antipsychotic activity (Sumner et al., 2004), so it is known to be altered by perturbations of DAergic signaling. Two other signaling cascade elements that were assayed, ERK1/2 and CREB, are key mediators of *c-fos* transcription (Fields et al., 1997; Ginty et al., 1997). In the present studies, both SUM and PPX decreased activation of ERK1/2 and CREB in ventral forebrain areas, with SUM typically having a more robust effect. However, at similar doses, only PPX reduced c-Fos expression. Conceivably, PPX effects on *c-fos* activation may reflect its activity within an alternative signaling pathway - in addition to those involved in ERK1/2 and CREB phosphorylation - that is not similarly activated by SUM. Alternatively, activation of D2 receptors by SUM - in addition to stimulating ERK1/2 and CREB phosphorylation - might activate signal pathways that prevent the suppression of c-Fos expression. A role of D3 activation in the PPX-induced suppression of c-Fos expression is supported by the finding that blockade of the D3, but not D2 receptors prevents PPX-induced decreases in c-Fos labeling, mostly in the NAc, an area of high D3R density. A similar study of *in vivo* administration of the D3-preferential agonist 7-OH-DPAT also demonstrated inhibition of *c-fos* in the striatum (Ishibashi et al., 2002). The D3 antagonist U99194 was previously reported to increase *c-fos* expression in these forebrain areas (Carr et al., 2002). While U99194 did not increase in c-Fos labeling in the present study, this may reflect methodological differences from the Carr et al. (2002) study; for example, the time interval between U99194 injection and tissue collection was longer in the present study, to permit an assessment of U99194 on the c-Fos-reducing effects of PPX.

The behavioral profile of PPX in rats is dominated by suppression and sedation; for example, it reduces both locomotor activity and startle magnitude (Chang et al., 2010a, 2010b, 2011; Kitagawa et al., 2009; Lagos et al., 1998; Maj et al., 1997; Svensson et al., 1994a; Weber et al., 2008a, 2009b). Conceivably, the suppression of NAc DA signaling elements, including *c-fos* expression - might be part of the cause-effect relationship linking PPX to behavioral supression. In contrast, SUM is relatively less prone to inhibit either startle magnitude (Weber et al., 2010b), locomotor activity (McCall et al., 2005) or c-Fos expression (present study). Any simple connection between levels of behavioral activity and NAc c-Fos expression could not easily account for the effects of L741626, which also decreases locomotor activity in rats (Chang et al., 2011; Millan et al., 2004b), but increases NAc c-Fos expression (present study).

In summary, pharmacological stimulation of D3 or D2 receptors show similar effects on the activation of $G_{i/o}$ proteins, PKA, CREB, and ERK1/2 in forebrain areas including the NAC and mCPu. In contrast, we observed significant suppression of c-Fos expression in these same brain regions after administration of a D3-preferential, but not D2-preferential agonist. This c-Fos suppression in the NAC was sensitive to D3 antagonism, but not D2 antagonism. While these findings cannot be interpreted to suggest that reduction of NAc c-Fos expression is a "D3-specific" signal, they do suggest that there are factors intervening between signaling levels of ERK1/2 or CREB activation and *c-fos* induction that might be differentially affected by D3 vs. D2 agonists; in turn, these factors may be sites of dissociation between the intracellular signaling of D3 and D2 receptors, which may be targeted in future development of D3-selective compounds.

F. Acknowledgements:

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DISCUSSION:

The dopamine D3 receptor and sensorimotor gating: linking behavioral and intracellular actions of D3 activation

A. Overview of major findings:

1. Pramipexole effects on sensorimotor gating: Agonists at dopamine (DA) receptors are known to reduce prepulse inhibition (PPI) of acoustic startle, an operational measure of sensorimotor gating, across several mammalian species (Braff and Light, 2005; Davis et al., 2008; Geyer, 2006; Swerdlow et al., 2008b). The D3-preferential agonist pramipexole (PPX) also reduces PPI in rats after acute administration, and this effect is observed across multiple rats strains, in both males and females, and across different stimulus modalities (Chang et al., 2010b, 2011; Swerdlow et al., 2009a; Weber et al., 2008a, 2009b). In humans, acute administration of bioactive doses of PPX produces either no change or modest increases in PPI under testing parameters that elicit PPX-induced reductions of PPI in rats (Swerdlow et al., 2009a). In C57BL/6J mice, PPX does not alter PPI, despite evidence of bioactivity based on changes in other behavioral measures (Chang et al., 2010a).

The PPX-induced disruption of PPI in rats is stereospecific - elicited by the S-enantiomer that binds D3 receptors, and not by the R-enantiomer that has antioxidant but not D3-binding properties - and can be detected after local intracerebral infusion of the drug at D3-rich sites (Chapter 3). Testing parameters for eliciting

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PPI disruption after PPX treatment have been optimized for dose, timing, and stimulus conditions (Chang et al., 2010b, Weber et al., 2008a).

Through the characterization of PPX effects on PPI across mammalian species, we have identified some important distinctions from other DA agonists, suggesting a unique pharmacological profile for PPX. For example, unlike the mixed D1/D2-family agonist apomorphine (APO) or the indirect DA agonist amphetamine, PPX does not produce differential PPI-disruptive effects in non-pigmented Sprague-Dawley vs. pigmented Long Evans rat strains (Breier et al., 2010; Swerdlow et al., 2007; Weber et al., 2008a, 2008b). APO and amphetamine, unlike PPX, also reliably reduce PPI in mice (Caldwell et al., 2009; Chang et al., 2010a; Martin et al., 2008; Ralph-Williams et al., 2002, 2003; Russig et al., 2005; Semenova et al., 2008; van den Buuse et al., 2005; Yee et al., 2004). Human studies have found associations between personality measures of novelty-seeking, sensation-seeking, disinhibition, and extraversion and effects amphetamine effects on PPI that were not evident with PPX (Chapter 1; Hutchison et al., 1999; Swerdlow et al., 2009a; Talledo et al., 2009). Conceivably, these drug-specific effects of PPX on PPI across species reflect its distinct D3-preferential properties, compared to the effects of amphetamine and APO.

Other studies have compared the effects on PPI of different D2/D3 agonists in an effort to parse the roles of D2 vs. D3 receptor in regulation of PPI. Since PPX and several other D2/D3 agonists have no effect on PPI in mice (Chapter 8; Chang et al., 2010a; Ralph and Caine, 2005; Ralph-Williams et al., 2003), D3 knockout studies would not be suitable for addressing this question. "Knockdown" studies in rats using virally delivered

short-hairpin RNAs targeting DRD3 mRNA might provide a more informative strategy for dissociating D2 vs. D3 effects; in our experience, this technique is not yet optimal in drug-naïve adult rats (Chapter 11), though it has been used successfully in one study of cocaine-administered rats (Bahi et al., 2005). Indeed, the difficulties in implementing this technology *in vivo* have been noted by other (Smits and Cuppen, 2006), and though it has been several years since shRNA-mediated knockdowns have been reported in rats (Zhang, et al., 2003; Lai et al., 2004), there are relatively few confirmatory reports using this technique.

Without molecular techniques at our disposal, strategies that leverage the pharmacological profiles of specific DAergic compounds have nevertheless been informative in discerning the role of D3 receptors in regulation of sensorimotor gating. The PPI-disruptive effects of the D3-preferential agonists PD128907, 7-OH-DPAT, and PPX, and those of the D2-selective agonist sumanirole, were tested after blockade of D2 receptors with the D2-selective antagonist L741626 (Chapter 4). At doses producing comparable PPI deficits, PPX exhibited the least sensitivity to L741626 antagonism, suggesting that this drug/dose combination was the most D3-selective among the three D3-agonists available to us, and about 30 times less sensitive than sumanirole. With this behavioral evidence for a relative insensitivity to D2 blockade, PPX was used as a tool for probing the role of D3 receptor activation in the regulation of sensorimotor gating. The apparent D3-selectivity of PPX effects on PPI were also utilized to behaviorally screen two putative D3-selective novel compounds: WC10 and WC44 (Chapter 5, Weber et al., 2009b), developing a behavioral paradigm that could be used to screen other novel

compounds in the future. Importantly, this study also provided additional evidence that *in vivo* activity of D3-active compounds could be very different from what is observed *in vitro,* suggesting that high-throughput behavioral screens for putative D3-selective compounds will be important additions to the development of new D3-based pharmacotherapies.

2. Dissociating PPX effects on PPI from those on other behaviors: Before probing the intracellular D3 signaling pathways associated with the effects of PPX on sensorimotor gating, studies examined the effects of PPX on a number of other behavioral measures. PPX has been shown to reduce startle magnitude at the doses used in our studies of PPI, and importantly - via a number of complementary experimental methods - we were able to dissociate PPX effects on startle magnitude and PPI (Chapters 2, 8; Chang et al., 2010b; Weber et al., 2008a).

In mice, PPX does not alter PPI, but elicits biphasic changes in locomotor behavior, consisting of early hypolocomotion and later-onset hyperlocomotion (Chapter 8; Chang et al., 2010a). Studies in D3 knockout mice with and without L741626 pretreatment suggested that D3 stimulation modulates the time course of PPX-elicited biphasic locomotor effects in mice - extending the early hypolocomotor phase, and delaying the emergence of hyperactivity - but that these locomotor effects are not otherwise dependent on D3 receptors. Moreover, only the late-onset PPX-induced hyperactivity appears to be mediated by D2 receptor activation, and the early onset hypoactivity is neither D2 nor D3 dependent (Chapter 9).

When locomotor activity effects of PPX were examined in *rats*, there was again a biphasic effect characterized by early hypoactivity and later hyperactivity. As with mice, the late-onset hyperactivity effects of PPX in rats were opposed by D2-blockade with L741626 (Chapter 10, Chang et al., 2011). By demonstrating that the PPI-disruptive effects of PPX were identical, whether PPI was tested at time points corresponding to the trough and peak of PPX-elicited locomotor activity, it was possible to demonstrate a clear dissociation between the systems regulating these two PPX-modified behaviors (Chapter 10, Chang et al., 2011). Thus, the neural changes responsible for PPX effects on locomotor activity and PPI are distinct. This conclusion is further supported by the differential sensitivity of these behavioral changes to D2 antagonism, and by the detection of PPX effects on locomotor activity but not on PPI effects in mice.

In summary, we demonstrated that PPX effects on PPI can be dissociated from its effects on other behavioral measures, including startle magnitude and locomotor activity. Thus, findings of the anatomical and biochemical effects of PPX - elicited using the experimental parameters developed in these early behavioral studies - may be most relevant to D3 mechanisms of specific relevance to PPI, and not necessarily to any general role of D3 activation in the control of other behaviors.

3. Parsing D2 vs. D3 effects on intracellular signaling cascades: Through the use of various *ex vivo* techniques, the intracellular effects that may correspond to the observed behavioral effects of D3 receptor activation were identified. We observed that PPX activated $G_{i/o}$ proteins and inhibited activity of PKA, CREB and ERK1/2, as well as

expression of c-Fos in forebrain areas. Comparisons with the effects of the non-selective D2-family agonist apomorphine and the D2-preferential agonist sumanirole revealed that, among the observed intracellular changes, only c-Fos expression appeared to be selectively altered by PPX, and this effect was opposed by the D3-preferential antagonist U99194, but not L741626 (Chapter 12). These findings might have several possible explanations, for example:

1) PPX stimulates both D2 and D3 receptors; its impact on PKA, CREB and ERK1/2 signaling may predominantly reflect its impact on D2 receptors. In contrast, c-Fos expression may be more D3-sensitive, and its suppression by PPX (but not sumanirole) may reflect the impact of PPX via some alternative, D2-insensitive signal pathway that was not specifically examined in these studies.

2) D3-mediated signaling may overlap with D2-mediating signaling in some pathways - e.g. PKA, CREB and ERK1/2 - and thus PPX effects on these molecules may reflect both D2 and D3 activation; in contrast, c-Fos expression may be regulated at or beyond a point of divergence in the signaling effects of these two receptor subtypes.

3) There is some mechanism through which D2 receptor stimulation by sumanirole counteracts a suppression in c-Fos expression, despite decreases in activity of signaling pathways known to control *c-fos* expression. This inhibitory mechanism is not stimulated by D3 receptor activation, and thus c-Fos expression is suppressed by PPX.

Conceivably, understanding the differential impact of D2- vs. D3 stimulation on NAc c-Fos expression could aid in the identification of D3-specific intracellular targets. However, to move forward with the investigation of the role D3 receptor activity in the regulation of sensorimotor gating - and the intracellular substrates that regulate this process - some other properties of DA receptor physiology should be considered; in turn, these properties may aid in the interpretation of studies summarized in this thesis, and the design of future studies of the D3 system.

B. Other considerations:

1. DA receptor heterodimers: While the experiments reviewed above sought to dissociate behavioral and intracellular effects of D2 vs. D3 activation, a growing body of literature suggests that the functional consequences of D2- and D3-receptor stimulation may be impacted by the formation of DA receptor heterodimers. Homo- and hetero-dimerization occurs with many G-protein-coupled receptors (GPCRs) (Bouvier, 2001), and has been exhibited between different subtypes of DA receptors, both within and across the D1- and D2-like families, as well as between DA receptors and GPCRs of other neurotransmitters (Fuxe et al., 2005; Gines et al., 2000; Hillion et al., 2002; Rocheville et al., 2000). These findings are relevant to *in vivo* D3 functioning because D3 receptors are known to be coexpressed in some neurons with D2 (Diaz et al., 2000; Gurevich and Joyce, 1999; Joseph et al., 2002; Joyce, 2001; Stanwood et al., 2000; Surmeier et al., 1996) and D1 receptors (Aizman et al., 2000; Surmeier et al., 1992). Presently, there are two recognized modes of dimerization. In "contact dimers," the

receptor monomers are touching but the individual binding sites are preserved. In contrast, "domain-swapping" dimerization involves the rearrangement and creation of two novel binding sites, where the recognition site of one monomer is physically associated with the coupling site of another monomer (Bouvier, 2001; George et al., 2002; Gouldson et al., 1998; Guo et al., 2003).

For D2 and D3 receptors, assembly into heterodimers results in binding and coupling profiles that differ from those of the respective monomers and may involve domain-swapping, as simulated with D2/D3 chimeras (Scarselli et al., 2001). In studies of transfected cells, PPX binds to D3 > D2/D3 chimeras > D2 receptors, but recognizes a greater proportion of high-affinity bindings sites in cells coexpressing D2 and D3 receptors than in cells expressing D2 or D3 receptors alone. D2/D3 heteromers bound to PPX still appear to act through $G_{i/o}$ proteins, though the exact intracellular coupling properties of D2/D3 heteromers have not been identified (Maggio et al., 2003). Along with observed "domain-swapping", the finding that dimerized GPCRs can activate a single G protein through a combination of intracellular loops of both protomers (Han et al., 2009) suggests that interactions of ligands with D2/D3 heteromers may lead to intracellular changes that differ quantitatively and qualitatively from the effects of either receptor subtype monomers or homomers (Maggio and Millan, 2010).

Evidence of D1 and D3 heteromers has also been observed, and these heteromers may display synergistic "intramembrane receptor-receptor interactions;" these interactions may explain the counterintuitive ability of $G_{i/o}$-linked D3 receptor activation to potentiate the effects of G_s-linked D1 stimulation (Fiorentini et al., 2008; Marcellino et

al., 2008; Schwartz et al., 1998). This synergy of DA receptor subtypes that are traditionally believed to be functionally opposed (cf. Neve et al., 2004) introduces the possibility of very diverse intracellular consequences of activation or antagonism by DA-active compounds; such effects would be dependent on the regional and cell-type distribution of D1, D3, coexpressed D1 and D3, and coexpressed D1 and D3 that form functional heterodimers. Additionally, heterodimers may have diverse mechanisms of action, perhaps adopting more "D1-like" or "D3-like", or even novel intracellular effects depending on the nature of the receptor interactions.

In short, while the experiments in this dissertation used traditional pharmacological tools to attempt to parse the D2- vs. D3-contributions to the regulation of PPI in rats, the recognition of DA receptor subtype heteromers introduces a new level of complexity to the understanding of D2 and/or D3 signaling cascades, and to our conceptions of GPCR pharmacology in general. This increased complexity likewise offers opportunities for development of new compounds that exhibit nuanced differences in activity from currently available ones, offering hope that "smarter drugs" could eventually be designed.

2. Functional selectivity: In addition to heterodimer formation, another phenomenon that is garnering increased attention in the field of GPCR signaling research is "functional selectivity." Also referred to as biased agonism, agonist-directed trafficking of receptor stimulus, pleiotropy, or pluridimensional efficacy (Galandrin et al., 2007; Kenakin, 2008; Urban et al., 2007), functional selectivity occurs when different ligands

of the same receptor display differential activation of some signaling pathways over others; this concept challenges previously accepted definitions of agonists, partial agonists, inverse agonists, and antagonists. Nearly a decade ago, it was observed that a ligand for a single receptor could act as a full agonist at one function and as an antagonist at another (Kilts et al., 2002). This phenomenon has since been recognized in several receptors, and may be a universal property of GPCRs (cf. Urban et al., 2007).

Regarding DA receptor pharmacology, the recognition of functional selectivity could warrant a reassessment of the signaling profiles of all known drugs. For example, the antipsychotic aripiprazole, originally identified as a partial D2 agonist (Lieberman, 2004; Stahl, 2001; Tamminga, 2002), has recently been suggested to instead display D2 functional selectivity, largely based on the original *in vivo*/*ex vivo* data from the drug's developers (cf. Mailman, 2007). Other DAergic compounds are slowly being identified as functionally selective (cf. Mailman and Murthy, 2010), and this process may aid in the development of clinically effective derivatives. The emergence of this phenomenon helps to explain some of the variation in the effects observed with compounds from the same "class" (e.g. D3 agonists, etc.), beyond that which can be explained by differences in receptor subtype specificity; data from each unique drug could therefore add nonredundant information to our understanding of neurophysiological processes. Thus, for studies of behavioral and intracellular effects of PPX, comparisons with other DA-active compounds and even other D3-preferential agonists may have multiple interpretations. While functional selectivity adds yet another level of complexity to the task of using pharmacological tools to explicate the neurobiology of DA-mediated

behaviors, as with the discovery of heterodimers, this property of DA receptors provides additional opportunities for pharmacological diversity and increasingly sophisticated mechanisms for drug discovery.

C: Clinical Significance:

1. D3 receptors, antipsychotics and PPI: All antipsychotic medications functionally antagonize activity at D2-family receptors (Creese et al., 1996). Whether the therapeutic effects of antipsychotics would be preserved in the total absence of D2 binding is not known, but preclinical models that have been useful in screening potential antipsychotics can offer some predictive insight. The ability of antipsychotics to prevent PPI-disruptive effects of apomorphine strongly correlates ($r = 0.99$) with their clinical potency (cf. Swerdlow et al., 1994), making PPI a useful behavioral model for assessing novel therapeutics. D3-preferential agonists have been shown to disrupt PPI in rats (Caine et al., 1995; Swerdlow et al., 1998a; Varty et al., 1998; Zhang et al., 2007). The studies described in this dissertation suggest that PPX disrupts PPI in a manner that appears to be dissociable from D2 effects - perhaps to a greater degree than D3 agonists used in previous reports - supporting the contention that stimulation of D3 receptors alone may be sufficient to disrupt sensorimotor gating in rats. That the D3 receptor may normally regulate sensorimotor gating in humans is suggested by the finding that DRD3 Ser9Gly polymorphism is associated with PPI differences in healthy adults (Roussos et al., 2008a). Conceivably, the ability of antipsychotics to restore PPI in animal models,

and indeed in humans (cf. Swerdlow et al., 2008b), may reflect in part their action at D3 receptors.

In our studies of PPX and other DA agonists, c-Fos emerged as one signaling molecule that was differentially suppressed by preferential D2- vs. D3-agonists. *c-fos* is an immediate early gene that can be induced by diverse physiological and pharmalogical stimuli (Herdegen and Leah, 1998). Induction of *c-fos* is one of the earliest signs of a genomic response to a stimulus (Sheng and Greenberg, 1990), and has been widely used as a measure of neural activation (Herrera and Robertson, 1996; Hoffman and Lyo, 2002). In distinction to the c-Fos-suppressing effects of PPX in the present studies, clinically effective antipsychotics *increase* c-Fos expression in the rat NAc shell, which may be predictive of antipsychotic effects, while antipsychotic-induced c-Fos expression in the NAc core and caudo-putamen may be associated with liability for extrapyramidal symptoms (Arnt et al., 1997; Deutch et al., 1992; Dragunow et al., 1990; Hoffman and Donovan, 1995; Oka et al., 2004; Robertson and Fibiger, 1996; Robertson et al., 1994; Sumner et al., 2004; Suzuki et al., 2010). This anatomical distinction roughly corresponds to the distribution of D3 receptors in the brain. Findings described in this thesis, regarding PPX effects on both PPI and c-Fos expression thus serve to link the D3 system with behavioral and cellular profiles that have been useful in predicting antipsychotic therapeutic potential. Specifically, D2-independent PPX effects on PPI and NAc c-Fos expression suggest that NAc D3 receptors - rather than D2 receptors per se - can regulate these two processes that are commonly used to understand the mechanisms of antipsychotic action.

The present studies identified some evidence for dissociable effects of D2- vs. D3-stimulation on PPI and its underlying substrates at the levels of neurochemistry (pharmacology), neuroanatomy (regional specificity) and intracellular signaling pathways. Though the compounds applied in this process - particularly PPX, sumanirole, L741626, and U99194 - proved adequate for parsing the behavioral and cellular effects of D2 vs. D3 receptor activation, more selective compounds might be expected to further clarify the roles of DA receptor subtypes in mediating antipsychotic effects on PPI and DA signaling. In this process, the convergent use of behavioral and cellular measures to understand dissociable drug effects on DA receptor subtypes may facilitate the development and screening of novel compounds with therapeutic potential. Because D2-family antagonists are used to treat several different neuropsychiatric disorders, including Tourette Syndrome and Bipolar Disorder, this development of novel therapeutics will have clinical utility beyond the schizophrenias.

2. D3 receptors and clinical applications beyond schizophrenia: Although most of the studies reviewed here have been geared toward development of antipsychotics, there is a large body of literature evaluating the potential of selective D3 antagonists for treating drug addiction (cf. Heidbreder, 2008; Heidbreder and Newman, 2010). While this topic is beyond the scope of the current review and summarized thoroughly elsewhere, the mesolimbic distribution of D3 receptors makes them an attractive drug target for pharmacotherapy for drug addiction. Indeed, D3-selective antagonists have shown promising effects in animal models of drug addiction, and DRD3

polymorphisms have been associated with addictive behaviors (cf. Heidbreder and Newman, 2010).

Interestingly, the most common therapeutic applications of D3 receptors involve D3 agonists, like PPX. PPX is currently approved for the treatment of Parkinson's Disease (PD) (cf. Antonini et al., 2010) and restless leg syndrome (RLS) (Trenkwalder et al., 2008), and is used as a second-line treatment for a number of seemingly unrelated conditions, including depression (Aiken, 2007; Barone, 2011), Tourette Syndrome (Bestha et al., 2010) and fibromyalgia (Marcus, 2009). Some studies are exploring possible pro-cognitive effects of PPX. For example in one study, PPX is reported to improve response accuracy in PD patients with low performance on working memory (WM) tasks, while patients with high WM performance showed no improvement (Costa et al., 2009). Another study reported that a single dose of PPX could improve performance on a number of cognitive tasks in individuals with lower peripheral D3 mRNA (Ersche et al., 2011). Increasing evidence is emerging for the importance of the D2-family in cognition (Costa et al., 2009; Luciana et al., 1998; Mehta et al., 2004; Von Huben et al., 2006), including DRD3 (Ersche et al., 2011). Preclinical data from animals models also support the importance of the D3 system in cognition (Micale et al., 2010; Millan and Brocco, 2008; Millan et al., 2007) in a manner that is relevant to cognitive domains affected in schizophrenia (Young et al., 2009).

3. PPX and impulse control disorders: The density of D3 receptors in limbic regions of the brain may also contribute to some novel adverse effects attributed to D3

agonists. A subset of PD patients treated with PPX or other D2/D3 agonists such as ropinirole have been reported to develop symptoms of impulse control disorders (ICD) such as pathological gambling (cf. Gallagher et al., 2007; Dodd et al., 2005; Driver-Dunckley et al., 2003; Grosset et al., 2006; Molina et al., 2000), compulsive shopping (Giladi et al., 2007), hypersexuality (Giovannoni et al., 2000; Klos et al., 2005; McKeon et al., 2007; Munhoz et al., 2009) and compulsive eating (Nirenberg and Waters, 2006). This adverse effect cannot easily be attributed to DA receptor denervation supersensitivity in PD: unlike PD patients, individuals with RLS presumably do not have DAergic denervation, and yet may also exhibit similar adverse responses to D3 agonists, including compulsive gambling (Cornelius et al., 2010; Driver-Dunckley et al., 2007; Quickfall and Suchowersky, 2007; Tippman-Peikert et al., 2007).

The mechanisms underlying these adverse effects are being investigated in clinical populations as well as healthy controls through the use of laboratory tests designed to measure "impulsivity" (Hamidovic et al., 2008; Riba et al., 2008; Voon et al., 2010). D3 stimulation has been proposed to increase susceptibility to ICD-like behaviors through alterations of reward-related learning and reward prediction (Cools et al., 2006; Frank et al., 2007; Ye et al., 2010). Others suggest that the D3 agonist-induced adverse reactions do not reflect generalized increases in impulsivity, but are instead the manifestation of a new obsessive focus on one or a few specific "rewarding" behaviors (Ahlskog, 2011; McKeon et al., 2007). PD patients with ICD symptoms demonstrated decreased measures of impulsivity after PPX or ropinirole were discontinued, while non-ICD patients showed no change (Voon et al., 2010). In contrast, acute administration of

PPX to healthy participants does not stimulate gambling or delay-discounting behaviors (Hamidovic et al., 2008; Riba et al., 2008), and PPX even decreased impulsivity in one study (Ersche et al., 2011). Animal studies of "impulsivity" and gambling have attempted to model some of the clinical observations in rodents (Johnson et al., 2011; Madden et al., 2010), but this line of work is still in its early stages. Presumably, our ability to interpret the neurobiological basis of D3-linked impulsivity and other adverse drug effects will be enhanced by a clear understanding of the effects of D3 receptor activation on basic behavioral processes such as sensorimotor gating, and its underlying cellular and intracellular substrates.

D: Conclusions:

This dissertation describes a systematic approach to investigating D3 receptor physiology as it relates to the regulation of sensorimotor gating, employing behavioral, pharmacological, genetic, and cellular/molecular techniques in three different mammalian species. Studies characterized and developed tools that were subsequently applied to this work: the PPI-altering effects of numerous different compounds with differing D2- vs. D3-selective agonist and antagonist profiles were characterized for the first time, a high-throughput behavioral testing paradigm for screening novel D3-selective compounds was developed, and initial steps towards applying lentiviral vector-delivered shRNA technology to rat knockdown studies were described. Additional studies related the behavioral effects mediated by DAergic activation within forebrain areas to intracellular signaling changes, in order to test the *in vivo* validity of assertions about D3 receptor

physiology that had been obtained from *in vitro* experiments. While the significant structural homology between D2 and D3 receptors continues to be a challenge in selectively probing D3 receptor effects, studies demonstrated some strategies for parsing D2 vs. D3 receptor contributions to behavior and cell signaling, using tools that are currently available. Within the limits of resolution of these tools, findings suggest that a point of divergence in D2 vs. D3 signaling cascades may occur at the level of *c-fos* activation in the NAc. Given known associations between *c-fos* induction and antipsychotic potential, these findings may prove to be important in D3 receptor drug development. As our understanding of DA receptor functioning advances and the pharmacological and genetic tools available for studying this system become more sophisticated, it may be possible to utilize points of divergence - at experimental levels ranging from behavior to intracellular signaling cascades - to develop more effective and better tolerated therapeutics for schizophrenia and other brain disorders characterized by deficits in sensorimotor gating.

APPENDIX

Each chapter in this dissertation reflects the work of many people. The contributions made by the dissertation author to the studies in each chapter are briefly outlined below.

1. Pramipexole effects on startle gating in rats and normal men.

The author performed most of the animal experimentation and analysis of animal data. She also assisted with collection of data from human subjects.

2. Parametric approaches towards understanding the effects of the preferential D3 receptor agonist pramipexole on prepulse inhibition in rats.

The author designed these studies with the guidance of the second and senior authors and then performed all animal experimentation and wrote the manuscript.

3. Stereochemical and neuroanatomical selectivity of pramipexole

effects on sensorimotor gating in rats

4. Technical note: Pharmacological parsing of D2- vs. D3-mediated regulation of sensorimotor gating in rats.

The author designed these studies with the guidance of the third and senior authors and then performed all animal experimentation and wrote the chapter. This work will be presented as a poster that will be written by the author; because the conference limits the number of first-authored abstracts, the dissertation author will be listed as second author on this abstract and not the primary presenter.

5. Using prepulse inhibition to detect functional D3 receptor antagonism: Effects of WC10 and WC44.

The author assisted with animal experimentation and data analyses and also gave input during manuscript preparation.

6. Heritable strain differences in sensitivity to the startle gating-disruptive effects of D2 but not D3 receptor stimulation.

The author assisted with animal experimentation and data analyses and gave input during manuscript preparation.

7. The effects of the dopamine D2 agonist sumanirole on prepulse inhibition in rats.

The author assisted with animal experimentation and data analyses and gave input during manuscript preparation.

8. The effects of pramipexole on prepulse inhibition and locomotor activity in C57BL/6J mice.

The author formulated the hypotheses, designed the experiments with guidance, performed all animal experimentation, and wrote the manuscript.

9. The effects of pramipexole on locomotor activity in D3 mutant mice.

The author formulated the hypotheses, designed the experiments with guidance, performed all animal experimentation, and wrote the manuscript.

10. Disparate effects of pramipexole on locomotor activity and sensorimotor gating in Sprague Dawley rats.

The author formulated the hypotheses, designed the experiments with guidance, performed all animal experimentation, and wrote the manuscript.

11. Technical note: *In vivo* suppression of dopamine D3 receptor expression in adult rats using lentiviral vectors.

The author formulated the hypotheses and designed the experiments. She performed plasmid amplification and purification, animal surgeries, behavioral testing, and tissue collection. The dissertation author was the presenter of this work at two professional conferences and prepared the posters that were the basis of this chapter.

12. Signaling pathways of the D3 agonist pramipexole in the rat ventral forebrain.

The author formulated the hypotheses, designed the experiments, collected all data, and wrote the chapter. She will be preparing this poster for presentation at a professional conference and is the primary author for the abstract.

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