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The consequences of neurotensin deficiency on the behavioral effects of dopamine agonists and on striatal dopaminergic tone

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B.S., University of Georgia, 2004

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Abstract

The consequences of neurotensin deficiency on the behavioral effects of dopamine agonists and on striatal dopaminergic tone

By Lucy Guillory Chastain

Numerous lines of evidence have implicated the neuropeptide neurotensin (NT) in the pathophysiology of schizophrenia. Some schizophrenic patients show decreased cerebrospinal fluid concentrations of NT, a deficit which is normalized with antipsychotic drug treatment. In rats, enhancing NT neurotransmission produces antipsychotic-like effects on behavior and mesocorticolimbic dopamine system activity, leading to the hypothesis that NT may function as an endogenous antipsychotic drug. Utilizing mice lacking the NT gene (NT-/-), this dissertation sought to examine the consequences of NT deficiency on dopaminergic function and tone. Specifically, these studies examined 1) the behavioral effects of dopamine agonists on locomotion and sensorimotor gating and 2) dopamine concentrations and dopamine receptor and transporter expression and binding in terminal regions in adult NT-/- mice compared to wildtype (NT+/+) mice. Compared to male NT+/+ mice, male NT-/- mice showed a dose-dependent attenuation of acute hyperlocomotor response and decreased sensitization to the indirect dopamine agonist amphetamine. The disruptive effects of a selective dopamine D1-type receptor agonist on locomotor activity, startle amplitude, and prepulse inhibition were dose-dependently decreased in male NT-/- mice. Male NT-/- mice also showed altered behavioral responses to a selective dopamine D2-type receptor agonist, indicating altered D1-type and D2-type receptor function in the absence of NT. Male NT-/- mice had no changes in striatal and cortical dopamine or dopamine metabolite concentrations, but showed significantly increased dorsal striatal D2 receptor mRNA and increased D2-like binding densities in the caudate putamen and nucleus accumbens, a result that is consistent with observed increases in D2 levels in schizophrenics. Female NT-/- mice did not show altered locomotor responses to acute or repeated amphetamine administration, and did not show increased striatal D2-like densities compared to female NT+/+ mice. However, female NT-/- mice showed decreased D1-like densities in the nucleus accumbens, an alteration not observed in male NT-/- mice. In sum, NT deficiency alters striatal dopamine receptor function, expression, and binding, supporting an important, sex-specific role for NT in dopamine system development and function. These studies suggest an NT deficiency may contribute to the etiology of schizophrenia.

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TABLE OF CONTENTS

TABLES AND FIGURES

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BNST	Bed nucleus of the stria terminalis
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
COMT	Cathecol-*O*-methyl-transferase
CP	Caudate putamen
CSF	Cerebrospinal fluid
D1	D1 dopamine receptor
D2	D2 dopamine receptor
D3	D3 dopamine receptor
D4	D4 dopamine receptor
D5	D5 dopamine receptor
DA	Dopamine
DAT	Dopamine transporter
DOPAC	3,4-Dihydroxyphenylacetic acid
FCTX	Frontal cortex
HPLC	High pressure liquid chromatography
HVA	Homovanillic acid
i.p.	Intraperitoneal
IP_3	Inositol triphosphate
L-DOPA	dihydroxyphenylalanine
MAPK	mitogen-activated protein kinase
MAO	monoamine oxidase
NAcc	Nucleus accumbens

NN	Neuromedin N
NT	Neurotensin
NT-/-	Mice lacking the neurotensin gene
NT+/+	Wildtype mice
NTR	Neurotensin receptor
NTS1	Neurotensin receptor 1
NTS2	Neurotensin receptor 2
NTS3	Neurotensin receptor 3
NTS4	Neurotensin receptor 4
PAG	Periacqueductal gray
PFC	Prefrontal cortex
PKC	Protein kinase C
PPI	Prepulse inhibition
s.c.	Subcutaneous
S.E.M.	Standard error of mean
SN	Substantia nigra
SNP	Single nucleotide polymorphism
TH	Tyrosine hydroxylase
VP	Ventral pallidum
VTA	Ventral tegmental area

1. INTRODUCTION

1.1. NEUROTENSIN BACKGROUND

Neurotensin (NT) is a neuropeptide that was first isolated from bovine hypothalamus by Carraway and Leeman in 1973 (Carraway and Leeman, 1973). The amino acid sequence of NT is N-Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-C (Carraway *et al*, 1982). The NT gene is highly conserved among vertebrates and encodes a 169-170 amino acid precursor protein containing both NT and a closely related hexapeptide, neuromedin N (NN), which are cleaved from the precursor protein after translation (Bean *et al*, 1992; Dobner *et al*, 1987; Dobner *et al*, 2001; Evers *et al*, 1995; Kislauskis *et al*, 1988; Shaw *et al*, 1990). In rats, NT/NN gene expression is regulated by several *cis*-regulatory sequences including AP-1, cyclic AMP response, glucocorticoid response, and *c-jun* regulatory elements (Kislauskis and Dobner, 1990).

NT functions as a neurotransmitter and hormone and is present in the central and peripheral nervous system as well as the gastrointestinal tract, particularly the intestine (Reinecke, 1985). It has been implicated in a variety of physiological processes including vasoactivity (Bachelard *et al*, 1986; Carraway and Leeman, 1975), gastric motility (Zhao and Pothoulakis, 2006), appetite (Beck, 2000; Stanley *et al*, 1983), nociception (Dobner, 2006), thermoregulation (Bissette *et al*, 1982), anterior pituitary hormone secretion (McCann and Vijayan, 1992), and inflammatory response (Carraway *et al*, 1991; Castagliuolo *et al*, 1999). In the central nervous system (CNS) of rodents, NT cell bodies are located in the hypothalamus, hippocampus, amygdala, bed nucleus of the stria terminalis (BNST), lateral septum, substantia nigra (SN), ventral tegmental area (VTA), olfactory tubercles, striatum, basal forebrain, and periaqueductal gray (PAG) (Binder *et al*, 2001b; D'Este *et al*, 2007; Jennes *et al*, 1982; Mai *et al*, 1987; Smits *et al*, 2004; Uhl, 1982). In neurons, NT is stored in presynaptic vesicles and its release is Ca^{2+}-dependent (Bissette and Nemeroff, 1995). NT neurotransmission is terminated by cleavage of the peptide by peptidases (Checler *et al*, 1988).

There are four known NT receptors (NTRs). NTRs and their pharmacology are reviewed extensively in Kinkead and Nemeroff (2006b). This section summarizes and updates this review. The NTS1 receptor, a G protein-coupled receptor with the classic seven transmembrane spanning regions, is the best characterized. NTS1 is a levocabastine-insensitive receptor with high affinity for NT (Vita *et al*, 1993). NTS1 couples to $G_{q/11}$, $G_{i/o}$, and G_s, and modulates several second messenger systems. *In vitro* activation of NTS1 increases intracellular Ca^{2+} influx, and regulates cAMP, cGMP, inositol triphosphate (IP_3), and mitogen-activated protein kinase (MAPK) activity (Li *et al*, 2001; Skrzydelski *et al*, 2003; Slusher *et al*, 1994). NTS1 is present on glia and neurons in high amounts in the SN, VTA, lateral septum, BNST, lateral septum, and prefrontal, cingulate, insular, and suprarhinal cortices (Boudin *et al*, 1996; Cadet *et al*, 1993; Elde *et al*, 1990; Fassio *et al*, 2000; Pickel *et al*, 2001; Quirion *et al*, 1987; Tanji *et al*, 1999). In the mesocorticolimbic and nigrostriatal dopamine (DA) systems, it is located pre-synaptically on DA cell bodies and terminals as well as post-synaptically in the nucleus accumbens (NAcc) and prefrontal cortex (PFC) (Binder *et al*, 2001b; Quirion *et al*, 1985). In the striatum, it is known to modulate neuronal activity via allosteric receptor/receptor interactions with the DA 2 family (D2) receptors (Binder *et al*, 2001b), which will be discussed in detail in section 1.5. Studies suggest NT modulates several functions through NTS1 including the behavioral effects of amphetamine on sensorimotor gating and locomotion (Feifel *et al*, 1999b; Panayi *et al*, 2002), which will be discussed in sections 1.7 and 1.8.

NTS2 is also a G protein-coupled receptor with seven transmembrane spanning regions. NTS2 has a low affinity for NT and also binds the histamine H1 receptor antagonist levocabastine (Mazella *et al*, 1996). NTS2 is expressed on neurons and glia in the CNS (Nouel *et al*, 1999; Vita *et al*, 1998). NTS1 is coupled to G proteins that regulate phospholipase C, phospholipase A, and MAP kinase (Gendron *et al*, 2004). In

the CNS, high levels of NTS2 are located in the cerebellum, hippocampus, piriform cortex, and neocortex. Moderate levels are located in the hippocampus, PAG, caudate putamen (CP), and NAcc and low levels are located in the VTA and SN (Botto *et al*, 1997; Kinkead *et al*, 2006b). Studies suggest NT modulates nociception (Dobner, 2006; Maeno *et al*, 2004), sensorimotor gating (Feifel *et al*, 2010a), and fear memory (Yamauchi *et al*, 2007) through NTS2.

The two other identified NTRs are NTS3 (sortilin) and the putative NTR, NTS4 (SorLA/LR11). NTS3 and NTS4 are members of the family of Vps10p domain receptors with high affinity for NT (Jacobsen, 2001; Mazella *et al*, 1998). In contrast to NTS1 and NTS2, the NTS3 and NTS4 receptors are type I amino acid receptors with a single transmembrane spanning region. NTS3 is found in neurons and glia in the CNS and also in adipocytes (Lin *et al*, 1997). NTS4 is found in neurons in the CNS and also in testes, ovaries, and lymph nodes (Kanaki *et al*, 1998; Yamazaki *et al*, 1997). The majority of NTS3 and NTS4 receptors are found intracellularly and have been theorized to play a role in intracellular sorting processes (Mazella, 2001). NTS3 has also been posited to play a role in cell death, and NTS4 may play a role in terminating NT function (for review see Kinkead et al (2006b)). In addition to NT, there are several other structurally related endogenous peptides that bind to NTRs in a species-specific manner, including NN, xenin, xenopsin, LANT-6, contulakin-G, and kinetensin (for review see Kinkead et al (2006b)).

1.2 DOPAMINE BACKGROUND

DA is a catecholamine that functions as a neurotransmitter and neuromodulator in the CNS. It is synthesized in neurons from tyrosine, which is converted into dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH). L-DOPA is then converted into DA and stored in synaptic vesicles for release. Once released into the

synapse, DA neurotransmission is terminated by reuptake into the synapse by the DA transporter (DAT). It is broken down by cathecol-*O*-methyl-transferase (COMT) and monoamine oxidase (MAO). The major metabolites are 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (Elsworth and Roth, 1997). DA exerts its effects by binding to DA receptors. In mammals, there are two families of DA receptors: D1-like receptors, which include D1 and D5 receptors, and D2-like receptors, which include D2, D3, and D4 receptors. D1-like receptors are G_s or G_{olf}-coupled receptors which stimulate adenylate cyclase, and D2-like receptors are $G_{i/o}$-coupled receptors which inhibit adenylate cyclase. D1 and D2 receptors are the most abundant in the CNS. There are two isoforms of the D2 receptor: D2S, which exists pre-synaptically as an autoreceptor, and D2L, which exists post-synaptically (Neve *et al*, 2004). DA neurons show both slow, tonic (baseline) DA release, which is regulated by D2 autoreceptors, and fast, phasic DA release that is induced by cell firing (Grace, 1991).

DA cells are segregated into four major pathways in the brain (Bjorklund and Dunnett, 2007; Deutch, 1993). These four DA systems in the CNS include the: 1) nigrostriatal pathway, in which cells originate in the SN (A9) and retrorubral field (RRF) (A8) and project to the dorsolateral CP, 2) mesolimbic pathway, in which cells originate in the VTA (A10) and project to the NAcc, ventromedial CP, olfactory tubercles, septum, amygdala, BNST, and limbic cortices, 3) mesocortical pathway, in which cells originate in the VTA and project to the frontal cortex (FCTX),and 4) tuberoinfundibular pathway, in which cells originate in the preoptic and arcuate nuclei in the hypothalamus and project to the median eminence and posterior pituitary gland. The VTA also innervates the habenula and locus coeruleus. The nigrostriatal pathway (Fig. 1) is involved in motor control. The mesolimbic and mesocortical pathways (Fig. 1) (often grouped together as the mesocorticolimbic pathway) are implicated in motivation, reward, emotion, and

learning. The tuberoinfundibular pathway regulates prolactin secretion. In addition to these pathways, there are DA cell bodies located in the olfactory bulb and retina.

1.3 SCHIZOPHRENIA, DA, AND NT

Schizophrenia background

Schizophrenia is a profoundly debilitating psychiatric disorder characterized by disturbances in the perception of reality. It affects about 1% of the world population. Symptoms are divided into positive, negative, and cognitive dimensions. "Positive symptoms" are qualities that are normally not present and include auditory hallucinations, bizarre or paranoid delusions, and disordered thoughts. Negative symptoms are a loss of normal traits or abilities and include flat affect and emotion, poverty of speech, inability to experience pleasure, and lack of motivation. Problems with cognition may also be present such as short term memory and attention deficits (Freedman *et al*, 1991; Woo *et al*, 2009). Despite its prevalence, schizophrenia remains one of the most intractable psychiatric diseases. People with schizophrenia experience great mental and social handicaps, and the disease also places a great emotional and fiscal burden on their families. Individuals with schizophrenia have higher incidences of substance abuse, unemployment, and suicide than those without the disease (Brown *et al*, 2000). The challenge to better understand and treat schizophrenia remains one of the most pressing problems in psychiatry today.

The etiology of schizophrenia is complex and is thought to be a combination of genetic susceptibilities and environmental factors. There are several genetic polymorphisms associated with susceptibility to the disease (Mulle, 2012; Owen *et al*, 2004; Petronis, 2000; Straub and Weinberger, 2006). In addition, environmental insults that occur during development such as perinatal infections or birth complications (Bilbo and Schwarz, 2009; Pearce, 2001; Yolken and Torrey, 2008) as well as later life

emotional stress and/or drug abuse (Corcoran *et al*, 2003; Finlay and Zigmond, 1997; Holtzman *et al*, 2013; Howes *et al*, 2004; Large *et al*, 2011) are linked to increased risk of schizophrenia. It is theorized that genetic risk factors interact with these environmental insults to produce neurocognitive dysfunction and disruptions in neural circuits which may develop into psychosis (Tsuang, 2000; Tsuang, 2001).

Schizophrenia and the 'dopamine hypothesis'

Many studies suggest that the mesocorticolimbic DA system is disrupted in schizophrenia (Deutch, 1993). Early studies noted that administration of amphetamine, an indirect DA agonist that increases synaptic concentrations of DA in mesolimbic terminal regions, produced symptoms indistinguishable from the positive symptoms observed in schizophrenia. In addition, administration of low doses of amphetamine to schizophrenic patients exacerbated psychotic symptoms (Lieberman *et al*, 1987). Finally, all antipsychotic drugs act as antagonists at the D2 receptor producing decreased mesolimbic DAergic activity (Grace *et al*, 1997). Taken together, these observations suggest that dysfunctional hyperactivity in the mesolimbic DA system may be responsible for the positive symptoms of schizophrenia, a theory termed the 'dopamine hypothesis' (Lieberman *et al*, 1987; Meltzer and Stahl, 1976; van Rossum, 1966).

While the original 'dopamine hypothesis' remains useful, some evidence seems to contradict this theory, as clinical studies and translational experiments relevant to schizophrenia actually suggest a deficit in tonic (background) mesolimbic DA release with a compensatory upregulation of phasic (transient caused by cell firing) DA release (Carlsson and Carlsson, 2006; Grace, 1991). In addition, the original dopamine hypothesis did not account for the presence of negative and cognitive symptoms and the observed hypoactivity in the PFC of schizophrenics (Andreasen *et al*, 1997). Nonetheless, many imaging and post mortem studies have shown alterations in striatal

D2 receptor binding densities in the brains of schizophrenic patients, confirming a disruption in the mesolimbic DA system in schizophrenia (Seeman, 1987; Seeman and Kapur, 2000; Wong *et al*, 1997). Newer versions of the 'dopamine hypothesis' have refined the theory by proposing that, in addition to disrupted mesolimbic DA activity (possibly hyperactive phasic activity and hypoactive tonic activity), schizophrenia may involve hypoactivity in the mesocortical DA circuit, as well as alterations in other neurotransmitters within this circuit (Carlsson *et al*, 2001; Grace, 1991). Another challenge in understanding the etiology of schizophrenia is that it is unknown whether an altered mesocorticolimbic system is a primary dysfunction in schizophrenia or whether it is a compensatory alteration due to some other defect (Carlsson *et al*, 2001). Although theory on the role of the mesocorticolimbic circuit in the neurobiology of schizophrenia appears to be progressing, more research on the nature of disruptions within the mesocorticolimbic circuit relevant to schizophrenia is warranted.

NT system disruption in schizophrenia

Given its broad range of physiological functions, it is not surprising that NT has been implicated in a range of diseases including cancer (Carraway and Plona, 2006; Dupouy *et al*, 2011), inflammatory bowel disease (Karagiannides and Pothoulakis, 2008; Zhao *et al*, 2006), Parkinson's disease (Sadoul *et al*, 1984; Schimpff *et al*, 2001), Huntington's disease (Emson *et al*, 1985), autism (Angelidou *et al*, 2010), and drug addiction (Cáceda *et al*, 2006; Dobner *et al*, 2003). However, some of the strongest clinical and experimental evidence implicates altered NT neurotransmission in the pathophysiology of schizophrenia. NT concentrations in cerebrospinal fluid (CSF) have consistently been shown to be decreased in a subset of drug-free patients with schizophrenia (Breslin *et al*, 1994; Lindström *et al*, 1988; Manberg *et al*, 1985; Nemeroff *et al*, 1989b; Sharma *et al*, 1994; Widerlöv *et al*, 1982). Low levels of NT in CSF were positively correlated with severity of psychopathology including thought disorder, deficit

symptoms, disorganized behavior, and impaired functioning (Garver *et al*, 1991; Sharma *et al*, 1997). In this subset of patients, clinical improvement (especially in negative symptoms) was associated with a normalization of CSF NT concentrations after antipsychotic drug treatment.

Post mortem studies of brain tissue in schizophrenics have shown less consistent results with several studies showing no changes in NT levels in schizophrenic patients (Manberg *et al*, 1982; Palacios *et al*, 1991; Zech *et al*, 1986). However, some studies have found increased NT-immunoreactivity in the frontal cortex of schizophrenics (Manberg *et al*, 1985; Nemeroff *et al*, 1983b). Other studies have reported decreased NTR binding densities in the entorhinal cortex (Hamid *et al*, 2002; Wolf *et al*, 1995), caudate, cingulate cortex, and PFC (Lahti *et al*, 1998). Finally, single nucleotide polymorphisms (SNPs) in the non-coding regions of the NTS1 gene and the NT gene have been linked to schizophrenia. For the NTS1 gene, one SNP and one haplotype are associated with schizophrenia in the Han Chinese population (Ma *et al*, 2013). Preliminary data in our lab have shown a link between a SNP in the NT gene promoter region and increased sensorimotor gating disruption in African American patients with schizophrenia (Kinkead *et al*, 2008a). Interestingly, this SNP is associated with decreased gene transcription *in vitro*.

In addition to these clinical studies, animal studies utilizing central injection of NT and NTR antagonists have shown NT modulates the mesocorticolimbic DA system, sometimes producing physiological and behavioral effects similar to those produced by antipsychotic drug administration. These experiments are reviewed in the following sections. These studies suggest NT may actually function as an endogenous antipsychotic drug (Nemeroff, 1980).

1.4 ANATOMY OF NT WITHIN THE DA SYSTEMS

In rodent and human brains, NT cells and NTRs are located within all four DA systems (Bean *et al*, 1992; Emson *et al*, 1985; Quirion *et al*, 1987), but this review will focus on NT anatomy within the mesocorticolimbic and nigrostriatal DA systems as they are most implicated in the pathophysiology of schizophrenia (Deutch, 1993) and in the regulation of locomotor and sensorimotor gating behavior (Robinson and Becker, 1986; Swerdlow *et al*, 1986).

Mesocorticolimbic DA system

In the mesocorticolimbic DA system of rodents, a small portion of TH-positive cells in the VTA colocalize NT (Bean *et al*, 1992; D'Este *et al*, 2007; Seroogy *et al*, 1988; Seroogy *et al*, 1987). This colocalization of NT and DA within VTA cells occurs in both rats and mice although some studies have observed low expression of NT in VTA cells and lack colocalization with TH in certain strains of mice (D'Este *et al*, 2007; Smits *et al*, 2004). These NT/DA neurons project to the PFC, entorhinal cortex, NAcc, basolateral nucleus of the amygdala, BNST, and the lateral septum (D'Este *et al*, 2007; Fallon, 1988; Febvret *et al*, 1991; Seroogy *et al*, 1987) (Fig. 2). In addition, the VTA is innervated by NT projections arising from the lateral hypothalamus (Kempadoo *et al*, 2013; Leinninger *et al*, 2011; Zahm *et al*, 2001). There is dense NTS1 expression in the VTA and many NTS1 receptors are expressed on DAergic neurons (Cadet *et al*, 1993; Palacios and Kuhar, 1981; Smits *et al*, 2004). NTS2 and NTS3 receptors are also found in the VTA (Sarret *et al*, 1998; Sarret *et al*, 2003a).

Postsynaptically, in the mesocorticolimbic system, NT-expressing cells exist in the NAcc shell and core, lateral septum, amygdala, and BNST (Betancur *et al*, 2000; Roubert *et al*, 2004; Smits *et al*, 2004; Zahm, 1987). Accumbal NT cells are GABAergic projection neurons that project to the VP and lateral hypothalamus (Binder *et al*, 2001b; Castel *et al*, 1994a; Castel *et al*, 1994b; Diaz *et al*, 1994; Diaz *et al*, 1995; Merchant *et*

al, 1992; Zahm, 1992). Studies utilizing electron microscopic methods show that some accumbal NT cells are also aspiny interneurons, which may be GABAergic or cholinergic (Delle Donne *et al*, 1996). Accumbal NT cells express D2, D3, and probably D1 receptors (Delle Donne *et al*, 1996; Diaz *et al*, 1994; Le Moine and Bloch, 1996). As previously mentioned, accumbal cells receive NT innervation by both NT/DA afferents from the VTA and local NT cells, and they also receive NT afferents from another source, possibly the subiculum (Delle Donne *et al*, 1996; Fallon, 1988; Johansson and Folan, 1984). Notably, the NAcc shell contains more NT-positive cells than the core, which more closely resembles the CP organization (Pickel *et al*, 2001; Zahm, 1992).

There is also dense NTS1 binding in the NAcc, ventral pallidum (VP), and FCTX (Cadet *et al*, 1993). In the NAcc and FCTX, NTS1 is located pre- and post-synaptically (Delle Donne *et al*, 2004; Pickel *et al*, 2001). NTS2 is also found in the NAcc and FCTX (Mazella *et al*, 1996; Sarret *et al*, 1998; Sarret *et al*, 2003b), and NTS3 is found in the FCTX (Sarret *et al*, 2003a). In the NAcc shell, NTRs are expressed on spines, dendrites, cell bodies, axons, synaptic terminals, and glia (Delle Donne *et al*, 2004; Nicot *et al*, 1994; Pickel *et al*, 2001). Post-synaptically, in the NAcc shell, the NTS1 receptor is most often colocalized with the D2 receptor. Colocalization of NTS1 and D2 receptors on synaptic terminals also occurs, but is less frequent and is present in symmetric (most likely GABAergic axon collaterals and DAergic projections from the VTA) and asymmetric glutamatergic synapses arising from cortical areas, hippocampus, and amygdala (Delle Donne *et al*, 2004). Several experiments suggest a functional interaction between pre- and post-synaptic D2 and NTS1 receptors in the NAcc (Fawaz *et al*, 2009; Fuxe *et al*, 1992a; Fuxe *et al*, 1992b). These studies will be discussed in section 1.6.

Nigrostriatal DA system

In the nigrostriatal DA system, very few NT-positive cells are located in the SN (in contrast to the VTA, which shows dense NT immunoreactivity), and these cells are not colocalized with DA (Jennes *et al*, 1982; Seroogy *et al*, 1988; Smits *et al*, 2004; Uhl, 1982). Like the VTA, NT fibers originating from outside the midbrain also innervate the SN (Hökfelt *et al*, 1984; Woulfe and Beaudet, 1992). There is dense NTS1 receptor binding and expression in the SN and RRF, most of which occurs on DA cells (Cadet *et al*, 1993; Fassio *et al*, 2000; Nicot *et al*, 1995; Palacios *et al*, 1981; Smits *et al*, 2004). The NTS2 (Sarret *et al*, 1998; Sarret *et al*, 2003b; Walker *et al*, 1998) and NTS3 (Sarret *et al*, 2003a) receptors are also expressed in the SN.

Postsynaptically in the nigrostriatal system, NT cells and fibers are scarce. Unlike in the mesocorticolimbic system, there are no mixed midbrain NT/DA projections to the dorsal striatum (Seroogy *et al*, 1987). Without pharmacological manipulation, NT-positive cells in the dorsal striatum are found mostly in the ventromedial CP (Betancur *et al*, 2000; Merchant *et al*, 1992; Zahm, 1987). These NT-containing cells project to the globus pallidus (Eggerman and Zahm, 1988). However, following DA receptor antagonist administration, more NT-positive cells in the rat CP become apparent, and these cells project to the SN (Castel *et al*, 1993a; Zahm, 1992). Thus, these NT-positive cells are likely direct and indirect pathway spiny GABAergic projection neurons (Castel *et al*, 1994b). Despite relatively low numbers of NT-positive cells, NTR densities in the dorsal striatum are relatively high (Cadet *et al*, 1993; Quirion *et al*, 1985; Uhl, 1982). In the CP, NTS1 receptors are located presynaptically (Boudin *et al*, 1996). NTS2 and NTS3 receptors are also found in the CP (Sarret *et al*, 2003a; Sarret *et al*, 2003b).

1.5 EFFECTS OF NT SYSTEM MANIPULATION ON THE DA SYSTEMS

As reviewed in the previous section, NT is anatomically positioned to regulate the mesocorticolimbic and nigrostriatal DA systems. NT primarily antagonizes the effects of

DA at D2 receptors, but functional interactions between the NT and DA systems are complex and the net effect of NT on DA depends on the regional distribution of NT and DA receptors. For this reason, both the cellular and regional effects of NT on DA neurotransmission will be discussed in this section. Notably, some of the cellular and regional effects of NT resemble those of antipsychotic drug administration, leading to the hypothesis that NT may function as an endogenous antipsychotic drug (Nemeroff, 1980).

Cellular mechanism of action of NT on DA

Although other NTRs may be involved (Binder *et al*, 2001b), the effects of NT on DA via NTS1 are best characterized. When NT binds to the NTS1 receptor it can modulate DA neurotransmission via several distinct mechanisms: 1) internalization of the NT-NTS1 complex leading to regulation of TH gene expression, 2) alteration of DA cell firing via activation of second messenger cascades and ion channels, and 3) allosteric receptor/receptor interactions between the activated NTS1 receptor and DA D2-type receptors leading to functional antagonism of the D2 receptor. It is the last mechanism that has been compared to the mechanism of action of antipsychotic drugs, as all antipsychotic drugs are antagonists of the D2 receptor, and antagonism of D2-type receptors is thought to be essential to the efficacy of antipsychotic drugs (Deutch *et al*, 1991).

NT modulates DAergic tone through its regulation of TH gene expression. Once NT binds to NTS1 receptors, the NT-NTS1 complex is internalized (Hermans and Maloteaux, 1998). The complex then dissociates and is segregated into different trafficking pathways (Boudin *et al*, 1998; Hermans *et al*, 1997), and NT moves to the cell's nucleus and potentially regulates gene expression (Laduron, 1994, 1995). Specifically NT increases TH mRNA and protein in the SN *in vivo* and *in vitro* through an increase in transcriptional activity in the TH gene (Burgevin *et al*, 1992a; Burgevin *et al*, 1992b; Najimi *et al*, 2002).

NT increases firing in DAergic neurons via activation of NTRs on DA cells. This effect is mediated by an increase in conductance of a nonselective cation channel dependent on $G_{\alpha q}$ and/or $G_{\alpha 11}$ and IP_3 and a decrease in conductance of an inwardly rectifying K^+ channel (Ih) dependent on protein kinase C (PKC) (Binder *et al*, 2001b; Cathala and Paupardin-Tritsch, 1997; Wu *et al*, 1995a; Wu and Wang, 1995b). Using Ca^{2+} imaging and whole-cell patch clamp techniques, St-Gelais et al. (2004) showed NT-induced DAergic neuron excitation was dependent on Ca^{2+} influx. Using an NTS1 receptor antagonist, they also found this excitation was dependent on activation of the NTS1 receptor.

NT also modulates DA neurotransmission via allosteric receptor/receptor interactions between the NTS1 receptor and D2-like receptors. Specifically, through activation of NTS1, NT antagonizes the function of D2-like receptors. The precise mechanism for this phenomenon is becoming increasingly clear from recent studies. Early studies showed NT decreases the affinity of D2 receptors for DA and DA agonists (Agnati *et al*, 1983; Fuxe *et al*, 1992b; Liu *et al*, 1994; Tanganelli *et al*, 1993; von Euler *et al*, 1991). Farkas et al. (1997) found NT inhibits K^+ conductance induced by a DA agonist suggesting NT and DA have opposing actions on the same K^+ current. However, other studies have shown that more direct allosteric receptor/receptor interactions between the activated NTS1 receptor and DA D2-type receptors exist, leading to D2 receptor desensitization. Jomphe et al. (2006) showed activated NTS1 is able to reduce D2 function through a PKC- and Ca^{2+}-dependent mechanism. Recently, Thibault et al. (2011) showed NT is able to internalize and desensitize both D2 receptor isoforms via activation of the NTS1 receptor *in vitro.* Finally, a recent studies have shown the the existence of NTS1/D2 heteromers *in vitro* suggesting a direct interaction between the receptors might occur as a mechanism for D2 receptor desensitization (Borroto-Escuela *et al*, 2013; Koschatzky *et al*, 2011). While most studies have focused

on the role of NTS1 in desensitizing D2 receptors, it is possible that other NTRs or NT itself might be able to modify D2 sensitivity, as NT was shown to alter D2 receptor antagonist binding in a cell line lacking any NTRs (Mandell *et al*, 1998).

Regional effects of NT injection on DA

The regional effects of NT depend on the brain region's unique cell population and receptor distribution. Experiments utilizing central injection of NT, *in vivo* electrophysiology, and microdialysis have elucidated NT's effects in various areas of the mesocorticolimbic and nigrostriatal circuits. More recent studies have utilized new NTR agonists and antagonists, voltammetry, and gene knockout techniques to further investigate the systemic effects of NT on DA the DA systems. Interestingly, central injection of NT can resemble the effects of peripheral administration of either antipsychotic drugs or psychostimulants, depending on the injection site (Bérod and Rostène, 2002; Dobner *et al*, 2003; Kinkead and Nemeroff, 2002). Specifically, intra-VTA injection of lower doses of NT resembles the excitatory effects of psychostimulant administration on the mesolimbic DA system, while intra-NAcc injection of NT resembles the inhibitory effects of antipsychotic drug administration on mesolimbic DA activity.

Several studies have characterized the effects of NT administered centrally in the midbrain (Fig. 1-3). The effects of NT centrally administered in the midbrain are dose-dependent. At low, physiological concentrations, NT opposes DA autoinhibition and induces depolarization of DA neurons in the VTA and SN (Cador *et al*, 1989; Cathala *et al*, 1997; Farkas *et al*, 1996; Kalivas *et al*, 1983; Laitinen *et al*, 1990; Mercuri *et al*, 1993; Nalivaiko *et al*, 1997; Shi and Bunney, 1992; Steinberg *et al*, 1995; Wu *et al*, 1995a; Wu *et al*, 1995b). At higher doses, NT increases the number and rate of spontaneously firing DA neurons. Notably, these effects resemble the effects of acute administration of psychostimulants. In contrast, at high, non-physiological doses, NT induces depolarization inhibition of DA neurons, effects similar to those produced by chronic

administration of antipsychotic drugs (Binder *et al*, 2001b; Shi and Bunney, 1991). Intra-VTA injection of low dose NT increases DA concentrations and/or the concentrations of DA metabolites on midbrain terminal regions including the NAcc, PFC, septum, amygdala, olfactory tubercles, and diagonal band of Broca (Cador *et al*, 1989; Kalivas *et al*, 1983; Laitinen *et al*, 1990; Sotty *et al*, 2000). When NT is injected into the SN, DA and DA metabolite concentrations are increased in the CP and globus pallidus although to a lesser extent than in the NAcc (Blaha *et al*, 1990; Chapman *et al*, 1992). Notably, the effects of NT-mediated DA efflux in the NAcc are mediated by the NTS1 receptor, as mice lacking the NTS1 receptor have decreased DA efflux when NT is injected in the VTA, whereas in mice lacking the NTS2 receptor DA efflux is unchanged (Leonetti *et al*, 2004). Administration of the NTS1 receptor antagonist SR 142948A, also blocks DA efflux in the NAcc when NT is injected into the VTA (Leonetti *et al*, 2002). Thus, the effects of NT on midbrain DA cell firing and DA release are thought to be due to NTS1 antagonism of D2 autoreceptor inhibition via allosteric receptor/receptor interactions described previously (Binder *et al*, 2001b).

Experiments utilizing local injection of NT into midbrain DA terminal regions have also been conducted to elucidate the neurotransmitter's complex interactions with DA and other neurotransmitters (Fig. 1-4). When injected into the NAcc, NT has varying effects on DA cell firing (Beauregard *et al*, 1992; McCarthy *et al*, 1979; Stowe *et al*, 2005). Injection of low dose NT into the NAcc is associated with increased local GABA release and a subsequent decrease in DA release from VTA terminals (Binder *et al*, 2001b; O'Connor, 2001). GABA release is also increased in accumbal terminal regions including the VP. Notably, NT's inhibitory effects on DAergic neurotransmission in the NAcc resemble those produced by chronic antipsychotic drug administration. There is also evidence that endogenous NT down-regulates DA efflux in the NAcc, as administration of NTR antagonists enhances both haloperidol-induced facilitation of

electrically-invoked DA release and haloperidol-induced facilitation of basal DA release in the NAcc (Brun *et al*, 2001; Brun *et al*, 1995). The mechanism of the effects of NT in the NAcc (increased GABA and decreased DA release) is likely due to NTS1's action on D2 receptors located post-synaptically on GABAergic neurons. NTS1 and D2 receptors are colocalized on GABAergic accumbal neurons (Delle Donne *et al*, 2004; Pickel *et al*, 2001), and NTS1 has been shown to desensitize the D2 receptor *in vitro* (Borroto-Escuela *et al*, 2013; Thibault *et al*, 2011). Enhanced GABA release is thought to be mediated by antagonism of D2 receptors by NTS1 receptors located on GABAergic NAcc neurons. The observed decrease in DA release is blocked by the $GABA_A$ receptor antagonist bicuculine suggesting the decrease in DA release is due to presynaptic activation of the $GABA_A$ receptor (Binder *et al*, 2001b; O'Connor, 2001). It has also been suggested that NT may act on NTRs on cortico-accumbal glutamate terminals, enhancing glutamatergic release onto accumbal GABA neurons, thus exciting local GABA release and GABA release in the VP (Ferraro *et al*, 2007). Recent studies show NT increases glutamate release in different brain regions (Antonelli *et al*, 2007a; Ferraro *et al*, 2008). Thus, GABA release may be increased and DA release may be decreased by GABA neuron collaterals' inhibition of mesolimbic DA terminals (Tanganelli *et al*, 1994). Ferraro et al. (2007) also suggest NTRs on astrocytes in the NAcc may increase glutamate release and thus decrease DA release. The mechanism for NT's action on DA in the NAcc, thus, still needs to be clarified.

At higher doses, intra-NAcc NT increases DA release in the NAcc (Chapman *et al*, 1992; Ferraro *et al*, 1997). This effect is thought to be due to pre-synaptic NTR inhibition of D2 autoreceptors at DA terminals. Indeed, anatomical data shows NT and D2 receptors are located pre-synaptically (Delle Donne *et al*, 2004; Pickel *et al*, 2001) and biochemical data shows NTS1 can desensitize and form complexes with both pre-synaptic (D2S) and post-synaptic (D2L) isoforms of the D2 receptor (Borroto-Escuela *et*

al, 2013; Thibault *et al*, 2011). Using fast-scan cyclic voltammetry, Fawaz et al. (2009) found evidence that NT acts directly on D2 autoreceptors on DA terminals, as DA release was enhanced by NT when the DA neurons were stimulated by spike trains. Also in support of this hypothesis, Legault et al. (2002) found evidence of such an NTR-D2 receptor interaction using patch-clamp recordings of cultured DA neurons.

In the dorsal striatum, perfusion of NT into the CP results in an increase in DA levels (Ferraro *et al*, 1997; Fuxe *et al*, 1992a; Tanganelli *et al*, 1989). Dorsal striatal perfusion of NT blocks the effects of D2 agonists (Fuxe *et al*, 1992a) and this effect is mediated via the NTS1 receptor (Antonelli *et al*, 2007b; Diaz-Cabiale *et al*, 2002). Specifically, when intrastriatal NT was co-perfused with a D2 agonist, it reduced the normal inhibition of striatal and pallidal GABA release by the D2 agonist, and this effect was blocked by an NTS1 receptor antagonist. In contrast, intrastriatal NT enhanced the effects of a D1 agonist, resulting in increased GABA concentrations in the striatum and in the globus pallidus. (Antonelli *et al*, 2007b; Fuxe *et al*, 1992a). Thus, it is likely that NT increases DA signaling through NTS1 receptor antagonism of D2 receptors located pre- and post-synaptically in the dorsal striatum (Antonelli *et al*, 2007b; Binder *et al*, 2001b), and the net functional effect of NT injection into the striatum is antagonism of the D2 receptor and a shift in post-synaptic DA transmission to D1 receptor-mediated effects (Antonelli *et al*, 2007b).

NT also regulates striatal DARPP-32, a DA signal transduction molecule. Mice lacking DARPP-32 have altered responses to DA, drugs of abuse, and antipsychotic drugs (Fienberg and Greengard, 2000; Fienberg *et al*, 1998). As stated above, intra-striatal NT causes an increase in DA, which leads to increased phosphorylation of DARPP-32 at Thr34 by protein kinase A (PKA), which is then converted into a potent inhibitor of protein phosphatase-1 (Matsuyama *et al*, 2002). D1 receptor antagonists block this increase in phosphorylation. Intrastriatal NT also inhibits phosphorylation of

DARPP-32 at Thr75 which disinhibits PKA (Matsuyama *et al*, 2003). Thus, NT likely potentiates its signalling via the DA/D1 receptor/DARPP-32 signalling cascade.

The effects of NT applied directly into the PFC have also been studied. NT injection into the PFC has effects on DA both locally within the PFC and on PFC efferents and afferents. Local application of NT in the PFC has no effect on firing rate of DA neurons, but co-application of NT with DA in this region attenuates the inhibitory effects of DA (Beauregard *et al*, 1992). In this same study, NT blocked the inhibitory effects of a D1 agonist 100% of the time and blocked the effects of a D2 agonist 50% of the time. Injection of NT into the PFC also increases glutamate release at terminal regions *in vitro* and *in vivo* (Ferraro *et al*, 2011; Ferraro *et al*, 2000), possibly through interactions between the NTS1 receptor and the D2 and NMDA receptors (Tanganelli *et al*, 2012). As the PFC sends excitatory projections to the NAcc, NT-induced glutamate release may lead to subsequent modulation of accumbal DA activity. In addition, NT application in the PFC increases the firing rates of about half of the DA cells in the VTA, likely due to stimulation by excitatory cortical projections (Fatigati *et al*, 2000; Rompré *et al*, 1998).

Effects of NTR agonists and antagonists on the DA systems

Several NTR agonists and NTR antagonists have been recently synthesized for potential use as antipsychotic drugs. Both agonists and antagonists have been shown to modulate the mesocorticolimbic and nigrostriatal DA systems. Like NT, the NTR agonist JMV 449 also increases TH mRNA and protein *in vitro* through an increase in transcriptional activity in the TH gene (Najimi *et al*, 2002; Najimi *et al*, 1998). Administration of NT69L, a novel NT analog (Boules *et al*, 2006), produces an increase in medial PFC DA and acetylcholine efflux, an effect similar to atypical APD administration (Prus *et al*, 2007).

Acute systemic administration of SR 48692, an NTR antagonist, decreases extracellular GABA release in the striatum, (Chapman and See, 1996) and increases the number of spontaneously active VTA neurons (Santucci *et al*, 1997). Paradoxically, chronic administration of NTR antagonists produces some effects on DA neurotransmission that are similar to central injection of NT or antipsychotic drugs. Chronic treatment with an NTR antagonist increases TH mRNA and protein in the ventral mesencephalon and decreases basal extracellular DA and DA metabolites in the NAcc but not PFC *in vivo (Azzi et al, 1998).* Subchronic administration of SR 48692 also reduces spontaneous cell firing in the VTA, but not in the SN (Santucci *et al*, 1997). This effect was hypothesized to be due to depolarization block of DA cells in the VTA and was compared with the same phenomenon observed following chronic antipsychotic drug administration (Grace *et al*, 1997; Santucci *et al*, 1997). Blocking NT neurotransmission by NTR antagonists and by NT gene knockout also has effects on DA-mediated behaviors. These studies will be reviewed in Section 1.7. Clearly, the functional interactions between DA and NT are complex and region-specific, so that the effects of systemic manipulation of NT systems on the DA systems are not easily predicted.

1.6 EFFECTS OF DA SYSTEM MANIPULATION ON THE NT SYSTEM

As reviewed in the previous section, NT system manipulation has physiological effects on the mesocorticolimbic and nigrostriatal DA systems. Conversely, manipulation of the DA systems also alters NT neurotransmission. Particularly systemic administration drugs modulating DA, including both psychostimulants and antipsychotic drugs, alters striatal NT release and NT expression. In addition, studies blocking NT neurotransmission with NTR antagonists or NT gene knockout show NT plays a mediating role in some of the physiological effects of both psychostimulants and

antipsychotic drugs. Finally, long-term changes in the DA system, including genetic knockdown of DA-related genes and selective ablation of DAergic cells increases NT system plasticity.

Psychostimulants

In general, DA system activation via systemic administration of indirect DA receptor agonists increases NT neurotransmission (Binder *et al*, 2001b). Amphetamine, methamphetamine, and cocaine are psychostimulants and indirect DA receptor agonists that activate the mesolimbic and nigrostriatal DA systems. These drugs bind the dopamine transporter (DAT), and either cause reverse transport of DA (amphetamine and methamphetamine) or inhibit synaptic DA reuptake (cocaine), thus increasing synaptic DA concentrations. In rats and mice, systemic administration of acute amphetamine, methamphetamine, and cocaine induces rapid increases in NT mRNA, peptide concentrations, and release in the CP and NAcc, particularly the dorsomedial CP and NAcc shell (Betancur *et al*, 2000; Binder *et al*, 2001b; Letter *et al*, 1987b; Wachi *et al*, 1987; Wagstaff *et al*, 1996b; Zahm *et al*, 1998). Methamphetamine-induced increases in striatal NT are mediated via the D1 receptor (Letter *et al*, 1987a; Merchant *et al*, 1988; Wagstaff *et al*, 1996b) and NMDA receptor (Singh *et al*, 1990; Wagstaff *et al*, 1997), as blocking these receptors blocks the increases in NT. Notably, methamphetamine administration also induces increases in NT peptides in the SN, and striatal methamphetamine-induced increases in NT are restricted to direct pathway striatonigral neurons (Castel *et al*, 1993b; Castel *et al*, 1994b; Merchant *et al*, 1990). Chronic administration of methamphetamine or cocaine also increases striatal NT mRNA and peptide content (Betancur *et al*, 1997; Hanson *et al*, 1989; Letter *et al*, 1987b; Merchant *et al*, 1988). Interestingly, while chronic administration of methamphetamine in rodents produces increased striatal NT concentrations, post mortem analyses of the brains of human chronic methamphetamine users showed decreased levels of NT in the

caudate (Frankel *et al*, 2007). It was unknown if this decrease was a consequence of damage to NT-containing cells, however.

Studies showing the NT system is altered in response to psychostimulant administration imply NT may have a role in mediating the effects of psychostimulants. Indeed, experiments utilizing NTR antagonists in rats and mice and NT gene knockout in mice demonstrate NT has a direct role in mediating striatal physiological response to psychostimulants. Systemic administration of amphetamine induces distinct patterns of immediate early gene expression in striatal regions; specifically *c-fos* mRNA and Fos protein is increased in both the CP and NAcc within one hour of administration (Dobner *et al*, 2003). Blocking NT neurotransmission by NTR antagonists or by NT gene knockout, reduces amphetamine-induced Fos increases in the medial striatum (Fadel *et al*, 2006). In another study NT antagonist administration diminished amphetamine-induction of *c-fos* mRNA in the PFC, mediodorsal thalamus, and NAcc (Cáceda *et al*, 2012). These studies demonstrate NT mediates some of the physiological effects of amphetamine. Rodent studies also show NT modulates some psychostimulant-induced behaviors, which will be discussed in Section 1.7.

Selective DA receptor agonists and antagonists

Systemic administration of selective D1 receptor or D2 receptor agonists and antagonists also alter NT neurotransmission within the DA systems in rodents. The effects of selective D1-like receptor agonists on striatal NT in rodents are similar to those of indirect DA receptor agonists. Systemic administration of SKF-38393, a selective D1-like receptor agonist, induces increases in NT peptides and NT mRNA in the dorsal striatum and NAcc (Hanson and Keefe, 1999; Merchant *et al*, 1989b; Taylor *et al*, 1991). Selective D1 antagonists do not produce changes in striatal NT mRNA (Augood *et al*, 1991), and effects of D1 antagonism on striatal NT content are variable (Taylor *et al*, 1991; Zahm, 1992). The selective D2-like receptor agonist quinpirole increases NT

release in the CP and NAcc while decreasing NT peptide content in these regions (Taylor *et al*, 1991; Wagstaff *et al*, 1996a).

Antipsychotic drugs and selective D2 antagonists

D2-like receptor antagonists, including both selective D2 antagonists and antipsychotic drugs which antagonize the D2 receptor non-selectively, also alter NT neurotransmission in rodents. The selective D2 receptor antagonist eticlopride increases NT peptides (Zahm, 1992), and the selective D2 antagonist raclopride increases NT mRNA (Augood *et al*, 1991) in the CP. In general, systemic administration of antipsychotic drugs increases NT mRNA and NT peptides in the striatum (Kinkead *et al*, 1999). For example, systemic administration of the antipsychotic drug haloperidol, a strong D2 receptor antagonist, increases NT mRNA and NT peptide concentrations in both the ventral and dorsal striatum, specifically in striatopallidal neurons indicating haloperidol stimulates NT increases in the indirect striatal pathway (Augood *et al*, 1997; Brog and Zahm, 1996; Eggerman *et al*, 1988; Zahm, 1992). In addition, subchronic administration of antipsychotic drugs in rodents increases NT peptide concentrations and release in the NAcc (Kinkead *et al*, 1999). As with amphetamine, pre-treatment with NTR antagonists and NT gene knockout also diminishes antipsychotic drug-induced increases in Fos protein in the dorsolateral striatum (Dobner *et al*, 2001; Fadel *et al*, 2001). These studies show that the effects of antipsychotic drugs on the NT system play a role in some of the drugs' physiological effects in the striatum. In addition, these studies suggest NT may play a role in the efficacy of antipsychotic drugs, implicating NT in the pharmacological treatment of schizophrenia (Kinkead *et al*, 1999; Kinkead *et al*, 2005).

Other alterations in DA system neurotransmission

In addition to pharmacological manipulation of the DA receptors, manipulation of the DA systems by selective ablation of DAergic pathways and DA-related gene

knockout also alters the NT system. Three days following ablation of DA-containing cells in the VTA by 6-OHDA, a catecholamine-selective neurotoxin, NT-immunoreactivity in the dorsal striatum and VP is increased in rats (Zahm and Johnson, 1989). Similarly, ablation of nigrostriatal DA-containing cells by 6-OHDA increases NT-immunoreactivity in the CP (Merchant *et al*, 1989a). These studies suggest DA exerts tonic inhibition on striatal NT, as lesioning DA-containing cells seems to disinhibit NT expression (Merchant *et al*, 1989a). Thus, it might be hypothesized that developmentally-induced hyperdopaminergia in a mouse model might be accompanied by decreased NT neurotransmission. Mice lacking the DAT show a chronic hyperdopaminergic state, particularly increased striatal extracellular DA. Interestingly, these mice also show significantly increased NT gene expression in the SN, VTA, and lateral septum (Roubert *et al*, 2004). The discrepancy between these studies might again be due to regional differences in DA/NT interactions; specifically, the interactions between DA and NT tend to be antagonistic in the striatum, while DA may stimulate NT neurotransmission in the midbrain and vice versa. These studies demonstrate NT system plasticity as a consequence of more long-term alterations in DA neurotransmission, highlighting the tight coupling of the DA and NT neurotransmitter systems and the complexity of DA/NT interactions.

1.7 THE ROLE OF NT IN THE LOCOMOTOR RESPONSE TO PHARMACOLOGICAL ACTIVATION OF THE DA SYSTEMS

As reviewed in the last section, NT plays a role in mediating some of the physiological effects of drugs known to activate mesocorticolimbic and nigrostriatal DA systems, including both direct and indirect DA agonists (Binder *et al*, 2001b; Dobner *et al*, 2003). In addition, many studies have shown NT also modulates the behavioral effects of these of drugs. Particularly, studies utilizing central injection of NT or NTR

antagonists suggest NT mediates the effects of DA-modulating drugs on locomotor behavior. These studies are summarized in this section.

Spontaneous locomotor behavior

The nigrostriatal and mesocorticolimbic DA systems are a part of the basal ganglia 'motive circuit', which translates biologically relevant environmental and pharmacological stimuli into adaptive motor responses (Pennartz *et al*, 1994; Pierce and Kalivas, 1997). Within this circuit, the striatum integrates diverse inputs, including those from the midbrain, cortex, and hippocampus, to appropriate motor outputs, potentially stimulating or inhibiting locomotion. As summarized in the previous sections, NT-containing cells and NTRs are localized at several regions in this circuit, and NT is known to modulate DAergic function in this circuit in a region-specific manner. Similarly, the effects of central injection of NT on locomotion vary by brain region. Intracerebroventricular injection of NT decreases spontaneous locomotion, an effect similar to the behavioral effects of antipsychotic drug administration (Nemeroff *et al*, 1977). However, infusion of NT into the VTA excites DA neuron firing and increases spontaneous locomotor activity, and chronic intra-VTA infusion of NT augments the hyperlocomotor effects (Elliott and Nemeroff, 1986; Kalivas *et al*, 1983; Kalivas and Taylor, 1985; Shi *et al*, 1992). These effects are similar to those of psychostimulant administration. Microinjection of NT in the NAcc does not affect spontaneous locomotor activity, but does block the effects of stimulants. (These studies are summarized below.) Blockade of NT neurotransmission by NTR antagonist administration does not appear to modulate baseline locomotor activity, suggesting endogenous NT does not regulate spontaneous locomotion (Panayi *et al*, 2002). However, several studies in rats and mice suggest NT modulates the effects of direct and indirect DA receptor agonists on locomotor activity. These studies are summarized below.

Effects of indirect DA agonists on locomotor activity

In rodents, indirect DA agonists, like amphetamine, methamphetamine, and cocaine, dose-dependently produce increases in locomotor activity and increase striatal synaptic concentrations of DA. At low to moderate doses, these psychomotor stimulants produce increases in forward locomotion. At higher doses, these drugs produce decreases in locomotion and increases in stereotyped behaviors including rearing, grooming, sniffing, swaying, jumping, and mouth movements (Holtzman, 1974; Kuczenski and Segal, 1989; Kuczenski *et al*, 1991; Sahakian *et al*, 1975). The excitatory effect of acute administration of psychostimulants on locomotor behavior is thought to be due to increased striatal DA activity and activation of D1-like and D2-like receptors (Chen *et al*, 2007; O'Neill and Shaw, 1999; Pierce *et al*, 1997; Swerdlow *et al*, 1986). With increased DAergic activity in the striatum, there is decreased GABA release at pallidal terminals, which then increases inhibition of the penduncolopontine nucleus (PPN), which then disinhibits spinal motor neurons to increase locomotion (Fig 1-5). In addition, decreased inhibition of ventral pallidal projection neurons leads to increased inhibition of the mediodorsal thalamus and decreased excitation at thalamocortical terminals, which may lead to decreased activity in the PFC (Brudzynski *et al*, 1988; Mogenson *et al*, 1993; Pennartz *et al*, 1994; Swerdlow *et al*, 1986). Increases in horizontal locomotor activity by indirect DA receptor agonists are thought to be due primarily to increased DAergic activity in the ventral striatum (NAcc), while increases in stereotypies are due mainly to activation of the dorsal striatum (CP) (Costa *et al*, 2007; Kelly and Iversen, 1976; Kelly *et al*, 1975).

Repeated administration of these psychostimulants results in an augmentation of the hyperlocomotor response accompanied by lasting changes in neurotransmission in the mesocorticolimbic DA system, a phenomenon called sensitization (Pierce *et al*, 1997; Robinson and Kolb, 2004). Particularly, enhanced accumbal DA release and decreased cortical DA release have been observed in rats sensitized to amphetamine

(Pierce *et al*, 1997). Interestingly, these changes in neurotransmission parallel the circuit alterations implicated in the neurobiology of addiction and psychosis (Carlsson *et al*, 1999; Grace, 1991; Kalivas and Volkow, 2005; Pierce *et al*, 1997). Thus, this behavioral paradigm is used as a model for both the development of psychostimulant addiction and for the psychotic features of schizophrenia and also provides a way to examine DA system function and plasticity (Robinson *et al*, 1986; Robinson and Berridge, 2000). In addition, the ability of a drug to inhibit acute and sensitized hyperlocomotor response to amphetamine is often used as a test for antipsychotic-like activity of a drug (Geyer and Ellenbroek, 2003).

Several studies in rats and mice suggest NT modulates psychostimulant-induced increases in locomotor activity, but it is unclear whether NT facilitates or inhibits psychostimulant-induced hyperactivity. Intracerebroventricular and intra-NAcc injection of NT reduces hyperlocomotor activity produced by amphetamine and cocaine (Nemeroff *et al*, 1983a; Robledo *et al*, 1993) but does not affect amphetamine-induced stereotypies (Jolicoeur *et al*, 1983), suggesting an inhibitory role for NT in the locomotor-stimulating effects of these psychostimulants. Likewise, chronic peripheral administration of NTR agonists reduces amphetamine and cocaine-induced hyperactivity (Boules *et al*, 2001; Feifel *et al*, 2008). Finally, overexpression of the NTS1 receptor in the NAcc reduces amphetamine-induced hyperlocomotor activity and rearing, suggesting increased NT neurotransmission in the NAcc counteracts the locomotor activating effects of amphetamine (Cáceda *et al*, 2005). However, in contrast to its acute effects, chronic central infusion of NT actually potentiates amphetamine-induced hyperlocomotor activity (Norman *et al*, 2008). Studies utilizing acute and chronic blockade of NTRs also suggest endogenous NT may facilitate, rather than inhibit, the acute hyperlocomotor effects of these psychostimulants and sensitization to these drugs. Acute and chronic NTR antagonist administration has no effect on acute amphetamine-

induced hyperlocomotion or stereotypies (Cáceda *et al*, 2012; Casti *et al*, 2004; Panayi *et al*, 2002), but chronic administration of an NTR antagonist reduced acute cocaine-induced hyperlocomotion and rearing without affecting stereotypies (Betancur *et al*, 1998). Pre-treatment with NTR antagonists also reduces amphetamine sensitization (Costa *et al*, 2007; Costa *et al*, 2001; Panayi *et al*, 2002) and delays cocaine sensitization (Horger *et al*, 1994). These studies utilizing NTR antagonists suggest endogenous NT neurotransmission is not involved in amphetamine-induced stereotypies, but might be involved in both acute psychostimulant-induced hyperlocomotor activity and psychostimulant sensitization, a possibility which is investigated in the studies described in Chapter 2.

Effects of direct DA receptor agonists on locomotor activity

Like psychostimulants, direct DA receptor agonists also dose-dependently modulate locomotor activity, but through direct stimulation of D1-like and D2-like DA receptors in the striatum. Nonselective DA receptor agonists and selective D1 and D2 family agonists have varying effects on locomotor activity. Administration of apomorphine, a nonselective DA receptor agonist, inhibits locomotor activity at low doses and excites locomotor activity at high doses. The inhibitory effect is thought to be due to primarily striatal D2 autoreceptor stimulation at low doses, and the excitatory effect is thought to be due to post-synaptic striatal D1 and D2 activation (Costall *et al*, 1981a; Di Chiara *et al*, 1976; Imperato *et al*, 1988; Kelly *et al*, 1975; Skirboll *et al*, 1979). Apomorphine also produces stereotypies and climbing behavior (Costall *et al*, 1981b). Notably, inhibition of apomorphine-induced hyperlocomotion and climbing behavior is predictive of antipsychotic-like efficacy in drugs (Geyer *et al*, 2003). Quinpirole, a selective D2-like receptor agonist, has behavioral effects similar to apomorphine, producing hypolocomotor activity at low doses and hyperlocomotor activity at high doses in rats and mice (Eilam *et al*, 1992; Jung and Shim, 2011), although some studies in

mice do not observe hyperlocomotor effects of quinpirole (Halberda *et al*, 1997). Studies suggest quinpirole-induced hypolocomotor activity is due to stimulation of pre-synaptic D2 autoreceptors and hyperlocomotor activity is due to post-synaptic D2 receptors in the striatum (Imperato *et al*, 1988; Wang *et al*, 2000). Most studies report administration of selective D1 family receptor agonists have been shown to increase locomotor activity and rearing in rats and mice (Frau *et al*, 2012; Halberda *et al*, 1997; Jung *et al*, 2011) although some studies report decreases in locomotion (Eilam *et al*, 1992). The mechanism of D1 agonist-induced alteration of locomotor behavior is thought to be due to post-synaptic D1 receptor stimulation in the striatum.

Although there are scarce studies, some experiments suggest NT modulates the locomotor effects of direct DA receptor agonists. One study reported central injection of NT decreased apomorphine-induced hyperactivity, while another reported no change in this behavior (Jolicoeur *et al*, 1983; Nemeroff *et al*, 1983a). Both experiments reported central NT injection did not affect apomorphine-induced stereotypies. Central injection of NT also diminishes apomorphine-induced climbing (Jolicoeur *et al*, 1991). NTR antagonism blocks apomorphine-induced turning but does not affect apomorphine-induced hyperlocomotion, hypolocomotion, climbing, or stereotypies (Gully *et al*, 1995) suggesting endogenous NT does not modulate most of the locomotor effects of apomorphine. In addition, intrastriatal injection of an NTR antagonist also blocks turning behavior produced by apomorphine, a selective D1 receptor agonist (SKF-38393), and a D2-preferring agonist (bromocriptine) (Poncelet *et al*, 1994), indicating endogenous NT modulates turning behavior induced by striatal D1 and D2 receptor activation. The studies in Chapter 2 further investigate this possibility by examining the effects of selective DA receptor agonists in mice lacking NT.

1.8 THE ROLE OF NT IN THE DISRUPTION OF SENSORIMOTOR GATING

Prepulse inhibition

One of the hallmark features of schizophrenia is a disruption in sensorimotor gating, that is, the ability to efficiently filter the incoming flood of sensory stimuli. This primary deficit is thought to produce cognitive fragmentation in schizophrenics and may lead to positive symptoms like hallucinations. Some schizophrenic patients show deficits in prepulse inhibition (PPI) of the startle reflex, a measure of sensorimotor gating (Kumari *et al*, 2000). PPI is the ability of a weak stimulus (i.e., a weak, non-startling noise) to inhibit the motor response to a subsequent strong stimulus (i.e., a loud, startling noise). Although there is some variability, most healthy, non-schizophrenic subjects show inhibition in startle response when an acoustic pulse is preceded by a prepulse, whereas individuals with schizophrenia show deficits in PPI compared to non-schizophrenic controls. This deficit is normalized by antipsychotic drug treatment (Kumari *et al*, 1999).

The PPI paradigm has proven to be a valid animal model for sensorimotor gating deficits relevant to schizophrenia (Kilts, 2001; Swerdlow and Geyer, 1998). The circuitry mediating startle response is a simple trisynaptic circuit (Fig. 1.6); an auditory stimulus stimulates cells in the cochlear nucleus which project to the nucleus reticularis pontis caudalis (PnC), which then projects to spinal motor neurons enabling a startle response (Swerdlow *et al*, 2001). When a startling acoustic pulse is preceded by a prepulse, the prepulse is thought to activate the cochlear nucleus, which then excites the pendunculopontine nucleus (PPN), which inhibits the PnC, thus suppressing the startle response. This circuit is strongly regulated by forebrain circuitry, particularly the mesocorticolimbic DA system (Fig. 1.6), although the nigrostriatal DA system is implicated in PPI regulation as well (Swerdlow *et al*, 2001).

PPI can be pharmacologically disrupted in rodents by psychostimulants, including amphetamine, and by most direct DA receptor agonists (Geyer *et al*, 2001). PPI can also be disrupted by developmental manipulations, such as isolation rearing and neonatal lesions of the ventral hippocampus (Geyer *et al*, 2001; Swerdlow *et al*, 2000; Swerdlow *et al*, 2001). Finally, genetic manipulations such as selective inbreeding and gene knockout in rodents have produced PPI deficits (Geyer *et al*, 2002; Powell *et al*, 2009; Swerdlow *et al*, 2001). Several studies suggest NT neurotransmission is involved in regulating baseline PPI as well as disruption of PPI. These studies are summarized below.

Pharmacological disruption of PPI by DA agonists

Studies in rodents have shown the mesocorticolimbic DA system strongly regulates this circuit through VP projections to the PPN and thus modulates PPI (Fig. 1.6). Pharmacologically increasing mesolimbic DA system activity with direct and indirect DA agonists produces disruptions in PPI, while reducing mesolimbic DAergic activity with antipsychotic drugs restores disrupted PPI (Geyer *et al*, 2001; Swerdlow *et al*, 1990; Swerdlow *et al*, 1994; Swerdlow *et al*, 2001; Zhang *et al*, 2000). Drugs activating the mesolimbic DA system, like amphetamine or direct DA receptor agonists, decrease PPI in rats (Geyer *et al*, 2001) and mice (Dulawa and Geyer, 1996). Similar to locomotor disruption, amphetamine disrupts PPI by increasing synaptic DA concentrations in the NAcc, which leads to decreased GABA release at pallidal terminals, which then increases inhibition of the PPN. The PPN then disinhibits the PnC and spinal motor neurons, leading to decreased PPI (Fig. 1.6). Although the mesocorticolimbic DA system is the most implicated in regulating PPI disruption by DA agonists, the nigrostriatal DA system may also play a regulatory role (Swerdlow *et al*, 2001). Direct DA receptor agonists, like apomorphine, and some selective D1 and D2 receptor agonists also disrupt PPI via activation of striatal DA receptors (Geyer *et al*,

2001; Swerdlow *et al*, 2001). Notably, there is also evidence that that natural variability in striatal DA activity is related to individual deviations in PPI. In rats, baseline PPI is negatively correlated with extracellular accumbal DA and DOPAC concentrations, and low PPI is associated with low D2 autoreceptor sensitivity (Yamada *et al*, 1998).

Several studies implicate NT neurotransmission in modulating both baseline PPI and PPI disruption by DA receptor agonists. Some studies suggest that increasing NT neurotransmission enhances baseline PPI and counteracts the disruptive effects of DA receptor agonists on PPI. Systemic administration of NTR agonists enhances baseline PPI in mice similar to the effects of systemic administration of antipsychotic drugs (Feifel *et al*, 2010b). NTR agonists do not reliably affect baseline PPI in rats, but block the disruptive effects of amphetamine and dizocilpine on PPI (Feifel *et al*, 1999b; Shilling *et al*, 2003). Notably, NTR agonist administration does not block the disruptive effects of apomorphine on PPI however (Feifel *et al*, 1999b). In rats, infusion of NT into the NAcc increases PPI, similar to administration of antipsychotic drugs (Feifel *et al*, 1997; Geyer *et al*, 2001), while intra-VTA injection of NT has no effect on PPI (Feifel and Reza, 1999a). Finally, in rats overexpression of NTS1 receptors in the NAcc does not affect baseline PPI but blocks the disruptive effects of amphetamine in rats (Cáceda *et al*, 2005). These studies suggest activation of NT neurotransmission by injecting NT or overexpressing NTS1 receptors has antipsychotic-like effects, enhancing PPI and reversing the disruptive effects of some psychostimulants on PPI. They also pinpoint the NAcc as a probable site of action of NT's restorative effects on PPI.

While increasing NT neurotransmission tends to enhance PPI and block disruption of PPI, blocking NT neurotransmission has mixed effects on PPI. Studies utilizing NTR antagonists, show acute blockade of NT neurotransmission does not affect baseline PPI, but NTR antagonism does block the restorative effects of some antipsychotic drugs on PPI (Binder *et al*, 2001a). In contrast, other experiments utilizing

NTR antagonists suggest NT may also play a role in mediating the disruptive effects of some psychostimulants on PPI. Acute administration of NTR antagonists blocks the disruptive effects of amphetamine and dizocilpine on PPI, but enhances the disruptive effect of apomorphine (Cáceda *et al*, 2012). The discrepancy between the effects of NTR antagonism on PPI disruption of amphetamine compared to apomorphine is likely because the mechanisms of these drugs differ, in that amphetamine is an indirect DA receptor agonist and apomorphine is a direct D2-preferring receptor agonist. These studies paint a complex picture for the role of NT in PPI disruption, suggesting endogenous NT may either facilitate or block PPI disruption by DA receptor agonists, depending on the mechanism of the drug. To further probe this issue, the experiments in Chapter 2 investigate the role of NT in PPI disruption by utilizing selective DA receptor agonists and NT-/- mice.

Developmental and genetic disruption of PPI

In addition to pharmacological methods, several developmental and genetic manipulations produce PPI deficits in rodents. Decreased NT neurotransmission has also been observed in some of these models. Isolation rearing in rats produces deficits in PPI (Swerdlow *et al*, 1998) as well as disrupted NT neurotransmission (Binder *et al*, 2001a). Specifically, isolation-reared rats have decreased NT mRNA expression in the NAcc shell and increased NTR binding in this area as compared to their socially-reared counterparts. Different strains of inbred rats and mice show great variability in baseline PPI and sensitivity to PPI disruption (Geyer *et al*, 2002; Powell *et al*, 2009; Swerdlow *et al*, 2001; Swerdlow *et al*, 2005). In preliminary experiments in our lab, NT expression was found to be correlated with baseline PPI values among different strains of mice and rats. Specifically, among five different mouse strains, PPI was positively correlated with NT mRNA in the PFC (Kinkead and Nemeroff, 2006a). In another experiment, among three different rat strains, PPI was positively correlated with NT mRNA in the VTA

(Caceda *et al*, 2007). These studies demonstrate that, in rodents, individual differences in NT gene expression are correlated with PPI, and specifically, decreased NT expression is associated with decreased PPI.

Previous studies with NT knockout mice

Finally, male mice deficient in NT have sensorimotor gating deficits. (Kinkead *et al*, 2005). Mice lacking the NT gene (NT-/-) show an absence of NT gene expression and peptides (Dobner *et al*, 2001). In our lab, male NT-/- mice showed significantly decreased PPI and increased pulse alone startle amplitude compared to wildtype (NT+/+) mice. This result coincides with observations in some schizophrenic patients that show decreased NT concentrations in CSF. As PPI is often used as a model for sensorimotor gating deficits in schizophrenia, the observed PPI deficits in NT-/-, as well as the previously mentioned experiments showing a positive correlation between NT gene expression and PPI, suggest a deficit in NT may be a causal factor in the etiology of some schizophrenia symptoms. In addition, the PPI-enhancing effects of some antipsychotic drugs and the PPI-disruptive effects of amphetamine are diminished in NT-/- mice (Kinkead *et al*, 2005). These studies suggest that an intact NT neurotransmission is necessary for the PPI-modulating effects of amphetamine and some antipsychotic drugs. This theory is supported by the previously described studies showing NTR antagonism diminishes the PPI modulating effects of both antipsychotic drugs and psychostimulants (Binder *et al*, 2001a; Cáceda *et al*, 2012). In addition, as PPI is tightly regulated by the mesocorticolimbic DA system, and both amphetamine and antipsychotic drugs act on DA receptors in this system, this study may indicate developmental alterations in the DA system of NT-/- mice. This possibility is investigated in Chapter 3 and 4.

1.9 DISSERTATION RATIONALE AND GOALS

In sum, the NT and DA systems are anatomically and functionally intertwined. The functional interactions between DA and NT are complex, so that the effects of systemic manipulation of NT on the DA systems are not easily predicted. Studies blocking NT neurotransmission with NTR antagonists and enhancing NT neurotransmission with NTR agonists support a role for NT in modulating endogenous DA activity and the behavioral responses to drugs acting on the DA systems. Central injection of NT and administration of NT agonists often produces antipsychotic-like effects in rodent models. Finally, some schizophrenic patients show a deficit in CSF NT concentrations, which is normalized by pharmacological treatment.

In light of these studies, mice lacking the NT gene were previously generated by Dobner and colleagues (Dobner *et al*, 2001). These mice do not produce NT mRNA or NT peptides. Male but not female NT-/- mice were shown to have baseline PPI deficits and the effects of amphetamine on PPI were reduced in NT-/- mice (Kinkead *et al*, 2005). Notably acute NTR blockade does not induce PPI deficits in rats (Cáceda *et al*, 2012), suggesting NT-/- mice may have developed compensatory changes in the neural circuitry regulating PPI as a result of NT deficiency. As previously described, the mesocorticolimbic and nigrostriatal DA systems are known to regulate baseline PPI and amphetamine disruption of PPI (Fig. 1.6), and these DA systems are known to display a great deal of plasticity in response to genetic and developmental manipulations (Fauchey *et al*, 2000a; Gatzke-Kopp, 2011; Jones *et al*, 1998). Thus, it was hypothesized that NT-/- mice may have alterations in mesocorticolimbic or nigrostriatal DA system function and/or tone. This hypothesis was tested in the experiments described in this dissertation.

To examine DA system functioning in NT-/- mice, 1) the effects of repeated amphetamine administration on locomotor behavior and 2) the acute effects of selective

DA receptor agonists on locomotor behavior and sensorimotor gating (PPI) were tested in NT-/- and NT+/+ mice. In rodents, the effects of DA agonists on locomotor behavior and PPI are well characterized as is the underlying neurocircuitry involved in these behaviors (Fig. 1.5, 1.6). Thus these paradigms provide a good way to probe DA system sensitivity and function in NT-/- mice. In addition, DA agonist-induced hyperlocomotion, sensitization, and PPI disruption are useful as animal models of neurobiological and behavioral disruptions relevant to schizophrenia (Howes *et al*, 2004; Robinson *et al*, 1986; Swerdlow *et al*, 2000). To examine dopaminergic tone, we measured DA and DA metabolite concentrations, DA receptor and DAT gene expression, and DA receptor binding densities in mesocorticolimbic and nigrostriatal terminal regions in NT-/- mice compared to NT+/+ mice. In light of the sex differences observed in the effects of NT deficiency on baseline PPI, both male and female NT+/+ and NT-/- mice were used in these studies.

FIGURES

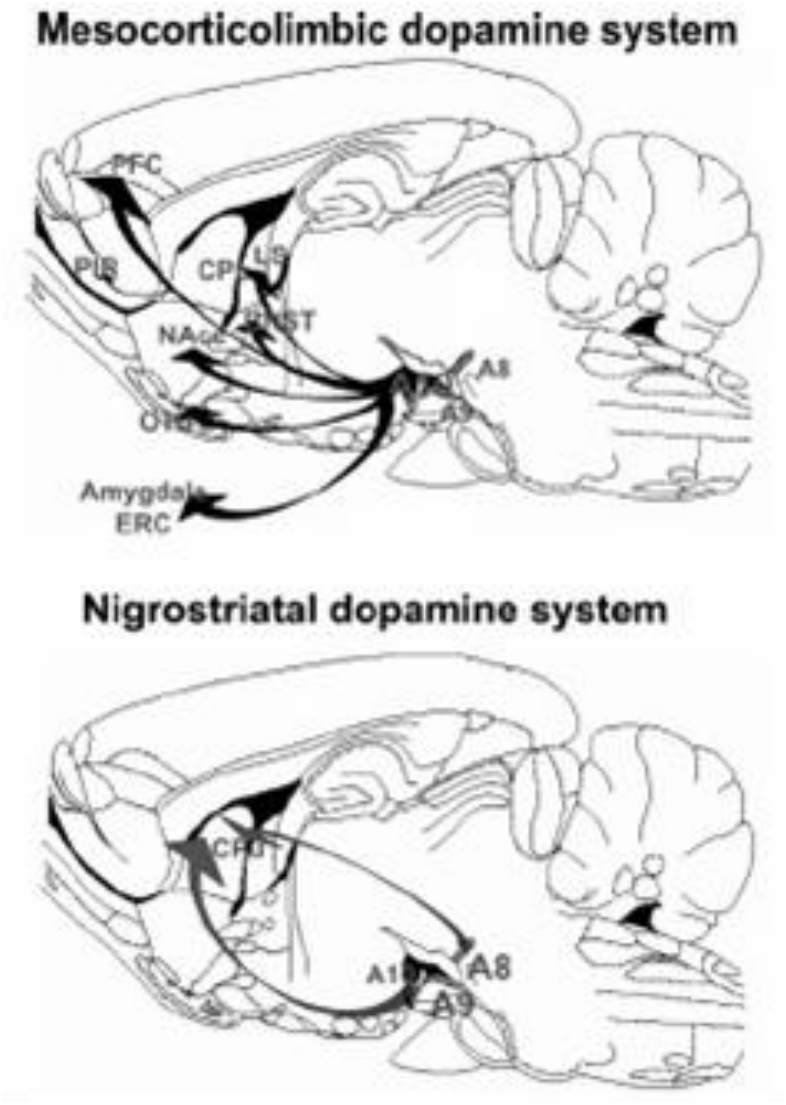

Fig. 1-1. Mesocorticolimbic and nigrostriatal DA systems in the rodent brain. Midbrain DA-containing cells send projections from A8 (retrorubral area), A9 (SN), and A10 (VTA) to striatal, cortical, and limbic areas (From Binder *et al*, 2001b).

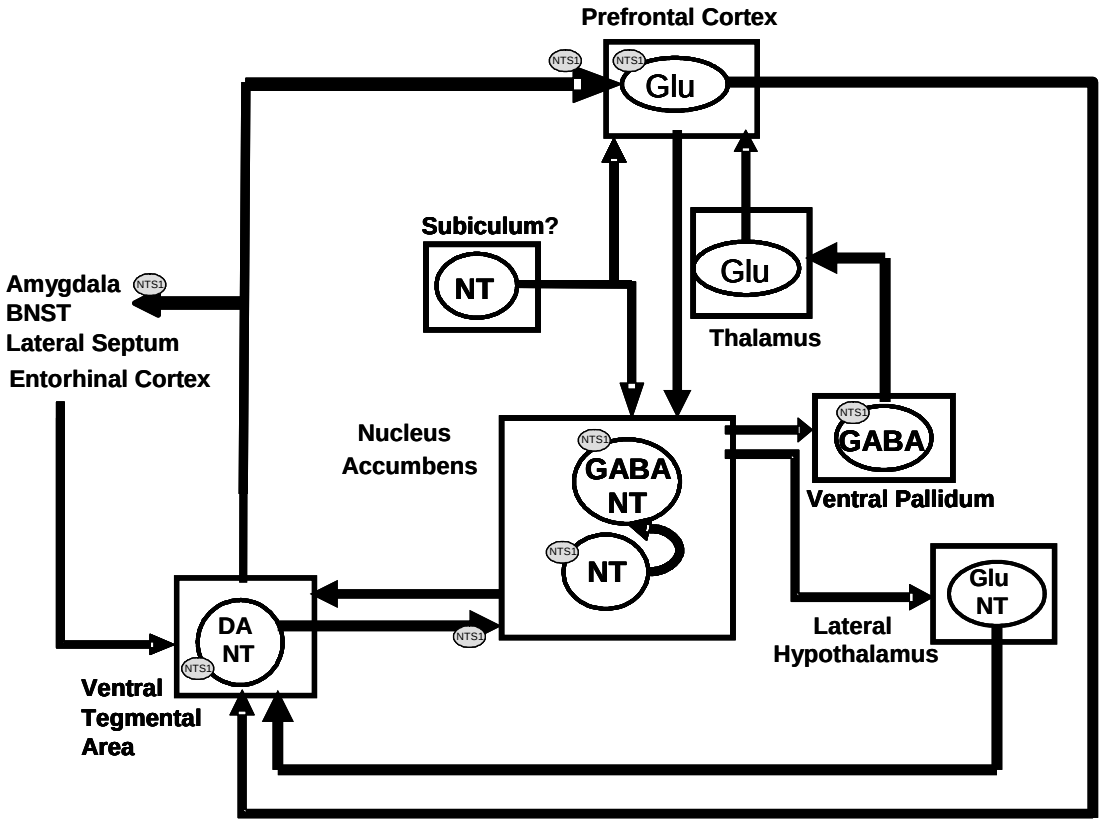

Fig. 1-2. NT-positive cells and NTRs within the rodent mesocorticolimbic DA system. The VTA sends NTergic efferents to the NAcc, prefrontal cortex, and other limbic regions. In the VTA, a small portion of DAergic cells colocalize NT, and these DA/NT cells project to the NAcc. NT-positive cells are also found in the NAcc, some of which colocalize GABA. NT receptors are found pre- and post-synaptically within the mesocorticolimbic system (Modified from Binder *et al*, 2001b).

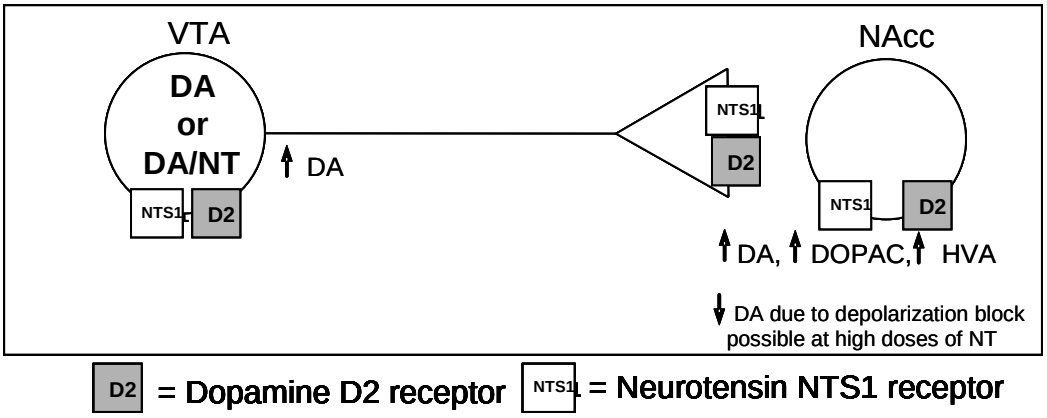

Fig. 1-3. The physiological effects of NT infusion into the ventral tegmental area (VTA) in rat. Intra-VTA injection of low doses of NT increases concentrations of DA and DA metabolites on terminal regions like the NAcc, effects similar to those produced by systemic administration of psychostimulants. Injection of high doses of NT decreases DA release and causes depolarization block, effects similar to those produced by systemic antipsychotic drug administration. These effects are thought to be mediated by NTS1 antagonism of D2 autoreceptors (Modified from Binder *et al*, 2001b).

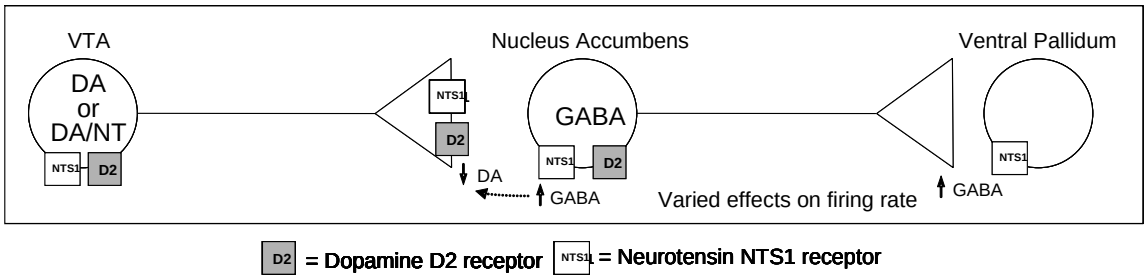

Fig. 1-4. The physiological effects of intra-accumbal injection of NT in the rat. Intra-NAcc injection of NT produces an increase in local GABA release and a decrease in DA release. GABA release is also increased in the pallidal terminal region. These effects are thought to be mediated by NTS1 antagonism of D2 receptors. Notably, the effects of intra-NAcc NT injection are similar to those produced by systemic administration of antipsychotic drugs (Modified from Binder *et al*, 2001b).

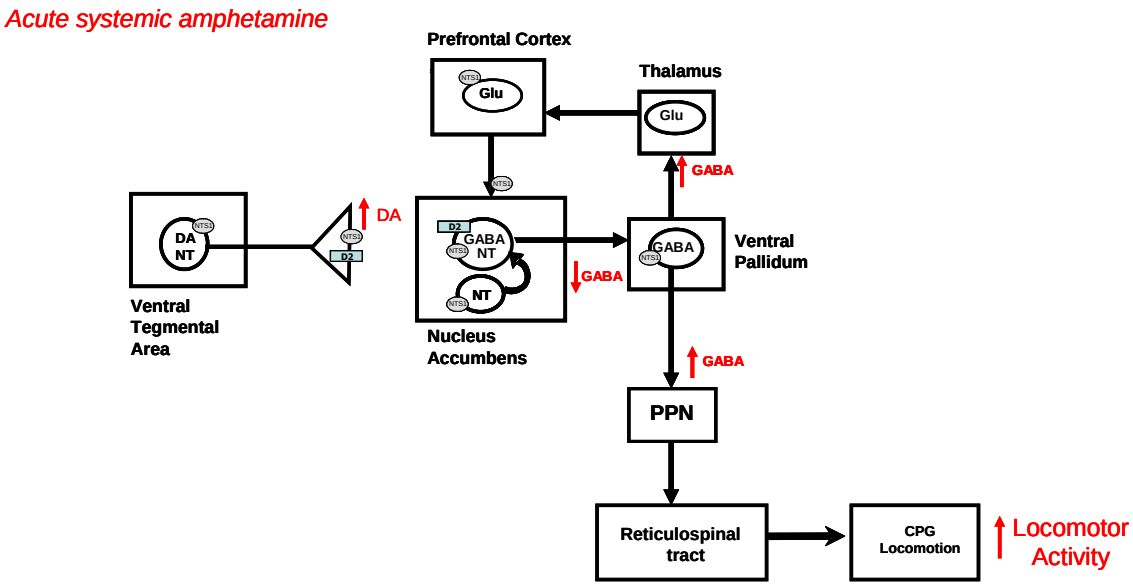

Fig. 1-5. Mechanism of mesolimbic modulation of amphetamine-induced hyperlocomotor activity. Systemic amphetamine administration leads to an increase in mesolimbic DA release and a subsequent decrease in GABA release at pallidal terminals. This results in increased inhibition of the penduncolopontine nucleus (PPN), which then disinhibits spinal motor neurons to increase locomotion (Binder *et al*, 2001b; Kinkead *et al*, 1999; Mogenson *et al*, 1993; Pierce *et al*, 1997; Swerdlow *et al*, 1986).

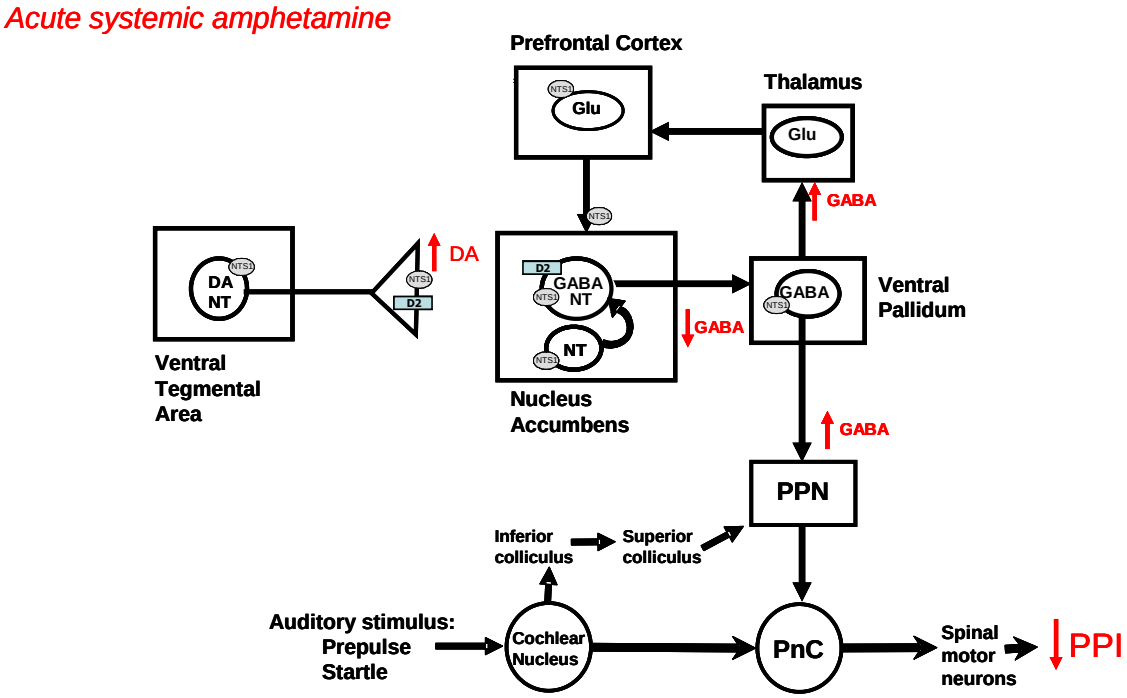

Fig. 1-6. Mechanism of mesolimbic modulation of amphetamine-induced PPI disruption. When a startling acoustic pulse is preceded by a prepulse, the prepulse is thought to activate the cochlear nucleus, which then excites the pendunculopontine nucleus (PPN), which inhibits the PnC, thus suppressing the startle response. Systemic amphetamine administration leads to an increase in mesolimbic DA release and a subsequent decrease in GABA release at pallidal terminals. This results in increased inhibition of the PPN, disinhibition of the PnC, and decreased PPI (Binder *et al*, 2001b; Kinkead *et al*, 1999; Swerdlow *et al*, 2001).

2. THE CONSEQUENCES OF NEUROTENSIN DEFICIENCY ON THE BEHAVIORAL EFFECTS OF DOPAMINE AGONISTS ON LOCOMOTION AND SENSORIMOTOR GATING

2.1 ABSTRACT

NT is a modulator of DA function and has been implicated in various DA-mediated behaviors including sensorimotor gating and locomotion. Compared to wildtype (NT+/+) mice, mice lacking the NT gene (NT-/-) were previously shown to have baseline deficits in PPI, a measure of sensorimotor gating highly correlated with mesolimbic DA function. The current studies investigated the consequences of NT gene knockout on the behavioral effects of dopaminergic activation by examining the behavioral effects of direct and indirect DA agonists in NT-/- mice compared to NT+/+ mice. Compared to NT+/+ mice, NT-/- mice showed a dose-dependent attenuation of acute locomotor response to amphetamine and diminished locomotor sensitization to repeated administration of amphetamine, while amphetamine-induced stereotypies did not differ between genotypes. These studies suggest that endogenous NT modulates the acute hyperlocomotor effects of amphetamine and amphetamine sensitization, and they also suggest an alteration in DA system function in the absence of NT. In order to independently assess the function of D1-type and D2-type receptors in NT-/- mice, the effects of the selective D1-family agonist SKF-82958 and the selective D2-family agonist quinpirole on locomotor behavior and PPI were investigated. The disruptive effects of the D1 agonist on locomotor activity, startle response, and PPI were dose-dependently decreased in NT-/- mice demonstrating D1-type receptor function is diminished in the absence of NT. At the higher dose tested (1 mg/kg), NT-/- mice showed a reduced hypolocomotor response to quinpirole. At the lower dose tested (0.1 mg/kg), quinpirole had no effect on startle amplitude or PPI in NT+/+ mice, but increased startle amplitude and PPI in NT-/- mice, showing altered D2-type function in the absence of NT. These studies demonstrate NT modulates the function of both D1 and D2 receptors. In the absence of NT, D1-type function is blunted and D2-type function is altered.

2.2 INTRODUCTION

The peptide NT modulates DA system function (Binder *et al*, 2001b), and several studies implicate NT in the physiological and behavioral responses to drugs modulating DA release and availability, particularly psychostimulants (Cáceda *et al*, 2012; Fadel *et al*, 2006; Kinkead *et al*, 2005) (see Introduction for review). Amphetamine, a psychostimulant and an indirect DA agonist, binds the DAT and causes reverse transport of DA, thus increasing synaptic DA concentrations. Systemic administration of amphetamine produces hyperlocomotion and disrupted sensorimotor gating (as measured by PPI) in mice and rats (Geyer *et al*, 2001; Geyer *et al*, 2002; Mansbach *et al*, 1988) by increasing mesolimbic DAergic activity (Mogenson *et al*, 1993; Pennartz *et al*, 1994; Swerdlow *et al*, 2001; Swerdlow *et al*, 1986). Repeated administration of amphetamine results in an augmentation of the hyperlocomotor response accompanied by lasting changes in the mesolimbic DA system, a phenomenon called amphetamine sensitization (Pierce *et al*, 1997). The amphetamine sensitization paradigm is often used as a model for both the development of psychostimulant addiction and for the psychotic features of schizophrenia (Robinson *et al*, 1986; Robinson *et al*, 2000). Chronic amphetamine administration paradigms also allow examination of DA system function and plasticity.

Studies blocking NT neurotransmission using NTR antagonists have suggested NT is necessary for some, but not all, of the behavioral effects induced by amphetamine. NTR antagonist administration and NT gene knockout block amphetamine disruption of PPI, but NTR antagonism does not affect the acute hyperlocomotor response to amphetamine (Cáceda *et al*, 2012; Casti *et al*, 2004; Panayi *et al*, 2002). These studies suggest NT is essential for the effects of amphetamine on PPI but not locomotor behavior. The reason for the distinction between the roles of NT in amphetamine-induced disruption of PPI and locomotion is unknown. However, NTR antagonists do

attenuate locomotor sensitization to repeated amphetamine administration, suggesting that NT plays a role in plasticity in the amphetamine-induced hyperlocomotor response (Costa *et al*, 2007; Costa *et al*, 2001; Panayi *et al*, 2002). Notably, conclusions drawn from studies utilizing NTR antagonists are limited because, while acting as antagonists at some NTRs (specifically NTS1), some compounds may act as agonists at other NTRs (NTS2) (Vita *et al*, 1998).

In contrast, other experiments suggest enhancing NT neurotransmission opposes the behavioral effects of amphetamine on locomotion and PPI. Particularly, systemic administration of NTR agonists blocks the disruptive effects of amphetamine on locomotor behavior and PPI (Boules *et al*, 2001; Feifel *et al*, 2008; Feifel *et al*, 1999b). One explanation for these seemingly contradictory studies could be that systemically administered NTR antagonists are primarily acting on different brain regions from systemically administered NTR agonists. (As detailed in the Introduction, NT shows opposing behavioral effects when injected in the NAcc compared to the VTA (Cáceda *et al*, 2006)). Nonetheless, from these studies, the role of NT in the behavioral effects of amphetamine remains unclear.

The purpose of these studies, in part, was to clarify the role of endogenous NT in the effects of acute and repeated amphetamine on locomotor behavior. Namely, 1) Does NT modulate acute amphetamine-induced hyperlocomotion? and 2) Does NT modulate sensitization to the hyperlocomotor effects of amphetamine? These experiments utilized mice lacking NT (NT-/- mice) to answer these questions. Utilizing NT-/- mice provides a means of investigating the role of NT in amphetamine response while avoiding the confounds of using NTR antagonists. NT-/- mice were previously shown to have baseline deficits in PPI (Kinkead *et al*, 2005). In addition, the disruptive effect of amphetamine on PPI was reduced in NT-/- mice (Kinkead *et al*, 2005). Experiment #1 investigated the acute hyperlocomotor response to amphetamine and

sensitization to amphetamine in NT-/- mice compared to NT+/+ controls. Experiment #2 examined the effects of amphetamine on stereotyped behavior in NT-/- mice compared to NT+/+ mice. Given the results from the previous studies using NTR antagonists, it was hypothesized that NT-/- mice would have a normal hyperlocomotor response to amphetamine but amphetamine sensitization would be blocked.

In addition to investigating the role of NT in the locomotor effects of amphetamine, these studies were also designed to interrogate DA system functioning in the absence of NT by utilizing NT-/- mice. While investigating the effects of amphetamine in mice lacking NT is very informative, the conclusions that can be drawn from these studies about the mechanism for the loss of function in NT-/- is limited. Amphetamine disrupts locomotor activity and PPI by increasing synaptic DA concentrations in the striatum, leading to increased activation of both pre-synaptic and post-synaptic DA receptors of both D1 and D2 families. The disruptive behavioral effects of amphetamine on PPI are thought to be dependent on the D2 receptor (Ralph-Williams *et al*, 2002b; Ralph *et al*, 1999), while locomotor sensitization to amphetamine is dependent on the D1 receptor (Vezina, 1996).

These experiments were designed to determine whether 1) In the absence of NT, is D1 receptor function altered? and 2) In the absence of NT, is D2 receptor function altered? In order to answer these questions, Experiment #3 investigated the effects of a selective D1-type receptor agonist (SKF-82958) and a selective D2-type receptor agonist (quinpirole) on locomotor activity and PPI in NT-/- mice compared to NT+/+ mice. The behavioral effects and mechanisms of action of SKF-82958 and quinpirole have been well characterized in rats and mice by previous studies. Like amphetamine, SKF-82958 produces hyperlocomotion and disrupts PPI in mice and rats (Frau *et al*, 2012). Quinpirole can either cause hypolocomotion or hyperlocomotion depending on the dose and the length of time following administration (Eilam *et al*, 1992; Horvitz *et al*,

2001; Jung *et al*, 2011; Luque-Rojas *et al*, 2012). Quinpirole disrupts PPI in rats (Peng *et al*, 1990; Wan *et al*, 1994), but has no effects on PPI in mice (Ralph-Williams *et al*, 2003; Ralph-Williams *et al*, 2002b). Given the results of previous studies employing NTR antagonists (Poncelet *et al*, 1994), it was hypothesized that the disruptive behavioral effects of SKF-82958 would be blunted in NT-/- mice. Given the results of previous NT central injection studies and the inhibitory actions of NT on the D2 receptor (Shi and Bunney, 1990; Shi *et al*, 1991), it was hypothesized that the disruptive effects of quinpirole in NT-/- mice would be enhanced.

2.3 METHODS

Animals

NT-/- mice were generated as previously described (Dobner *et al*, 2001). Mice (60 days of age and older) from the lab's NT-/- breeding colony backcrossed against the C57BL/6J strain were used for these studies. Only male mice were used in the experiments in this chapter. NT+/- mice were bred to generate wildtype (NT+/+) mice and mice lacking the NT gene (NT-/-). Animals were housed in an environmentally-controlled animal facility with a reversed 12 hour light-dark cycle (lights off at 10:00 AM; lights on at 10:00 PM). Food and water were available *ad libitum*. Mice were weaned on postnatal day 21 and housed in same sex groups of two to six per cage. All behavioral testing and euthanasia procedures were completed in the dark phase between 10:00 AM and 5:00 PM. All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee (IACUC) in compliance with the National Institutes of Health.

Genotyping

At weaning, ear punches were obtained from all mice and DNA was extracted from the tissue. The presence or absence of the NT gene was identified using custom PCR

primers (Invitrogen) to amplify the wildtype NT gene or the disrupted NT gene construct. Primer sequences to detect the wildtype NT gene allele were 5'-CATCCCTCACAGTTCACTCACTTTG-3' (25 mer, T_m=74°C) and 5'-CCTGGATTCATTTACCTGAGTAGCA-3' (25 mer T_m=72°C). Primer sequences to detect the NT-/- gene allele were 5'-CATCCCTCACAGTTCACTCACTTTG-3' (25 mer, T_m=74°C) and 5'-CCCAGTCACGACGTTGTAAAACGAC-3' (25 mer, T_m=76°C). The PCR products for the wildtype NT gene and for the NT-/- gene allele were 270 bp and 188 bp, respectively. PCR products were run on gel electrophoresis to identify the genotype for each animal.

Drug Administration

All drugs were dissolved in 0.9% saline and injected at a volume of 1.0 ml/kg. Mice were weighed before each testing session to determine the appropriate dose for each animal. *d*-Amphetamine sulfate was purchased from Sigma (St. Louis, MO), and was injected *s.c*. SKF-82958 hydrobromide was obtained through the NIMH Chemical Synthesis and Drug Supply Program (RTI International, Research Triangle Park, NC), and was injected *i.p.* Quinpirole was purchased from Sigma, and was injected *i.p.*

Startle response and PPI testing

Startle response and PPI were measured in San Diego Instruments (San Diego, CA) startle chambers as described in (Binder *et al*, 2001a). Startle amplitude was measured from vibrations of a Plexiglas cylinder (resting on a platform) caused by whole-body response. Vibrations were converted into analog signals using a piezoelectric unit attached to the platform. These signals were digitized and stored in a personal computer. The testing session began with a 5 min acclimatization to the startle chamber in the presence of 65 dB background white noise. Testing sessions consisted of eleven 120 dB pulses alone, eleven no stimulus trials, and 18 pulses preceded (100 msec) by a

prepulse of 4, 8, or 12 dB above background. Pulses were presented in a pseudorandom order with an average of 15 s between pulses. Percent PPI for each mouse at each prepulse intensity was calculated using the following formula: %PPI = 100 – (startle amplitude with prepulse x 100/startle amplitude with pulse alone.)

Locomotor testing

Locomotor activity measurements were evaluated by placing mice in an open field consisting of a white plastic bucket (24.5 cm in diameter, 26.5 cm in height) and videotaped under red light conditions. Activity was recorded for either 60 min (experiment #1 and #2) or 90 min (experiment #3), and videotapes were post-processed to quantify time-dependent spontaneous behavior. For experiment #1, distance moved by each animal in the arena was automatically determined using Ethovision 3.0 (Noldus Information Technology, The Netherlands). For experiment #3, distance moved by each animal in the arena was automatically determined using TopScan (Clever Sys Inc., Reston, VA).

Experiment 1

Effects of amphetamine on locomotor behavior

All animals underwent all tests and treatments in a within-subjects design. NT+/+ (*n*=8) and NT-/- (*n*=12) were first tested for baseline locomotor activity. A week after baseline testing, the effects of amphetamine (1 mg/kg *s.c.*) on locomotor activity were examined on two consecutive days (amphetamine treatment #1 and amphetamine treatment #2). Seven days after the 2nd treatment, animals received a third injection of amphetamine (amphetamine treatment #3) and locomotor activity was examined (experimental protocol summarized in Fig. 2-1). This experiment was repeated with another dose of amphetamine (2 mg/kg *s.c.*) utilizing another cohort of NT+/+ (*n*=7) and NT-/- (*n*=5) mice.

Experiment 2

Effects of amphetamine on stereotyped behavior

NT+/+ (*n*=7) and NT-/- (*n*=9) mice were administered 2 mg/kg amphetamine and were placed in an open field. For every animal, behavior was observed in 1 min samples once every 10 min for 60 min. Occurrence and frequency of stereotyped behaviors were recorded as detailed in Crawley et al. (1998). Behaviors to be recorded included; grooming, rearing, sniffing, swaying, jumping, yawning, licking, jaw tremor, biting, and self gnawing. For each animal, the frequencies of each behavior were summed across all the 1 min samples for a total frequency.

Experiment 3

Effects of SKF-82958 and quinpirole on locomotor behavior and PPI

In this experiment, all animals underwent all tests and treatments in a within-subjects design. NT+/+ (*n*=8) and NT-/- (*n*=13) mice first underwent baseline testing twice to obtain baseline PPI and locomotor values for each animal. For all tests, PPI testing was immediately followed by locomotor testing. For baseline testing, all animals received injections of 0.9% saline 10 min before testing began. One week after baseline testing, the effects of 0.1 mg/kg quinpirole on PPI and locomotor activity were examined. Mice were administered quinpirole (0.1 mg/kg *i.p.*), and 10 min later underwent PPI and locomotor testing. One week following this test session mice underwent PPI and locomotor testing with saline injections to verify baseline values. One week later, mice underwent four weeks of PPI and locomotor testing; each week the animals received an i.p. injection of 1 mg/kg quinpirole, 0.3 mg/kg SKF-82958, 1 mg/kg SKF-82958, or saline 10 min testing. All animals received each of these drug treatments, and the order of administration was counterbalanced.

Statistical Analysis

Data were tested for normality using the Shapiro-Wilk test. In all cases, the data passed the Shapiro-Wilk test, indicating the samples were from a Gaussian distribution. Thus, in all analyses, parametric statistical tests were utilized. For experiment #1 and #3, ANOVAs were utilized. Following ANOVAs, planned comparisons were tested using Tukey's HSD post-test.

For experiment #1, the two cohorts receiving separate doses were analyzed separately. Since the experiments utilized a within-subjects design, two-way repeated measures ANOVAs (genotype x drug) were used to analyze 1) the effect of acute amphetamine 2) the effect of repeated amphetamine administration on distance moved (m) during a 60 min time frame. Data from this experiment were also analyzed within each genotype by two-way repeated measures ANOVA (time point x drug treatment). For 1) the acute amphetamine analyses, Tukey's post-tests were used to compare the baseline session to the first amphetamine treatment session to measure acute amphetamine response. For 2) the subchronic amphetamine analyses, Tukey's tests were used to compare the first and second amphetamine treatment sessions to the third amphetamine treatment session to measure locomotor sensitization to amphetamine. For experiment #2, a Student's *t* test was used to compare the total frequency of each stereotyped behavior between genotypes.

For experiment #3, the behavioral effects of quinpirole and SKF-82958 on locomotor behavior, startle response, and PPI were analyzed. The effect of each drug dose on each behavior was analyzed separately. For each drug dose, the effects of genotype and drug dose (drug vs. saline control) were analyzed using two-way repeated measures ANOVAs. Tukey's post-tests were used for pairwise comparisons of saline controls to each drug dose and to compare genotypes. For locomotor tests, distance

moved (m) during three 30 min time windows was analyzed. Because baseline startle amplitude and PPI values were not statistically different from saline control values, the saline control and the two baseline tests were averaged to generate control values for startle amplitude and PPI comparisons. Significance was set at $p<0.05$ for all analyses. Statistical analyses were performed with GraphPad Prism 3.0 (GraphPad Software, San Diego, CA) and SysStat SigmaPlot 12.3 software.

2.4 RESULTS

Effects of acute and subchronic amphetamine on locomotor behavior in NT+/+ and NT-/- mice

For the cohort receiving 1 mg/kg amphetamine (Fig. 2-2a), a two-way repeated measures ANOVA (genotype x drug) for the acute amphetamine analysis showed a significant effect of drug ($F(1,18)=18.005$, $p<0.001$), no significant effect of genotype ($p>0.05$), and no genotype x drug interaction ($p>0.05$). Tukey's tests comparing the genotypes showed baseline locomotor behavior (distance moved) and locomotor response to the first amphetamine treatment were not significantly different between NT+/+ and NT-/- mice ($p>0.05$). Pairwise comparisons within the NT+/+ mice group showed the first amphetamine treatment (acute amphetamine) significantly increased distance moved compared to baseline ($p<0.01$). Within the NT-/- group, acute amphetamine also significantly increased distance moved compared to baseline ($p>0.05$). These results indicate there were no differences between NT+/+ and NT-/- in baseline locomotor behavior or acute locomotor response to 1 mg/kg amphetamine.

For the 1 mg/kg amphetamine sensitization analyses (Fig. 2-2a), a two-way repeated measures ANOVA (genotype x drug) showed a significant effect of drug ($p<0.001$). The effect of genotype ($p=0.056$) and the genotype x drug interaction ($p=0.069$) approached significance. Tukey's test within the NT+/+ mice and NT-/- mice

showed the third amphetamine treatment significantly increased distance moved compared to the first treatment ($p<0.05$), indicating both groups sensitized to the locomotor effects of amphetamine. Likewise, a two-way ANOVA (genotype x drug) comparing the second amphetamine injection (amphetamine #2) and the third amphetamine injection (amphetamine #3) showed a significant effect of drug ($p<0.001$), while the effect of genotype ($p=0.067$) and the genotype x drug interaction ($p=0.096$) approached significance. Again, Tukey's test within the NT+/+ mice and NT-/- mice showed the third amphetamine treatment significantly increased distance moved compared to the first treatment ($p<0.01$), indicating both groups sensitized to the locomotor effects of amphetamine. However, pairwise tests comparing the genotypes showed locomotor response to the third amphetamine treatment was significantly lower in NT-/- compared to NT+/+ mice ($p<0.05$). These results indicate sensitization to 1 mg/kg amphetamine was diminished in the NT-/- group compared to the NT+/+ group.

For the cohort receiving 2 mg/kg amphetamine (Fig. 2-2b), a two way repeated measures ANOVA (genotype x drug) for the acute amphetamine analysis showed the effect of drug ($F(1,10)=4.032$) approached significance ($p=0.072$). The effect of genotype was not significant ($p>0.05$) and the genotype x drug interaction approached significance ($F(1,10)=3.676$, $p=0.084$). Again Tukey*'s* tests showed baseline locomotor behavior (distance moved) was not significantly different between genotypes ($p>0.05$). However, locomotor response to the first injection of amphetamine by the NT-/- mice was significantly less than the locomotor response by NT+/+ mice ($p<0.05$) indicating NT-/- mice had a blunted locomotor response to acute amphetamine. Pairwise comparisons within the NT+/+ mice group showed the first amphetamine treatment (acute amphetamine) significantly increased distance moved compared to baseline ($p<0.05$). However, within the NT-/- group, acute amphetamine did not significantly

increase distance moved compared to baseline ($p>0.05$) indicating 2 mg/kg amphetamine did not produce a significant hyperlocomotor response in mice lacking NT.

For the 2 mg/kg amphetamine sensitization analyses (Fig 2-2b), a two-way repeated measures ANOVA (genotype x drug) showed a significant effect of drug ($F(1,10)=24.743$, $p<0.001$), no significant effect of genotype, and no genotype x drug interaction ($p>0.05$). Tukey's tests within the NT+/+ mice and NT-/- mice showed the third amphetamine treatment increased distance moved compared to the first treatment in both groups ($p<0.05$) indicating both NT+/+ and NT-/- mice sensitized to the locomotor effects of amphetamine. Post-tests also showed no differences between the genotypes ($p>0.05$). These results indicate sensitization to 2 mg/kg amphetamine was not different between NT+/+ and NT-/- mice.

Effects of amphetamine on stereotyped behavior in NT+/+ and NT-/- mice

Student's *t* tests showed no differences in frequency of grooming, rearing, or sniffing between NT+/+ and NT-/- mice ($p>0.05$) (Fig. 2-3). Other stereotypies, such as swaying, jumping, yawning, licking, jaw tremor, biting, and self gnawing, did not occur.

Effects of selective DA receptor agonists on locomotor behavior in NT+/+ and NT-/- mice

Baseline locomotor testing showed no differences in distance moved between NT+/+ and NT-/- mice ($p>0.05$). The effects of 0.1 mg/kg quinpirole, 1 mg/kg quinpirole, 0.3 mg/kg SKF-82958, and 1 mg/kg SKF-82958 on distance moved were analyzed separately by two-way repeated measures ANOVAs (genotype x drug) at 30 min time windows (Fig. 2-4 and Table 2-1). All results for experiment #3 are also summarized in Table 3.

For the 0.3 mg/kg SKF-82958 (Fig. 2-4a), there was a significant effect of drug at the 0-30 min time interval ($F(1,16)=7.221$, $p<0.05$), but not at the other two time intervals ($p>0.05$). There were no significant effects of genotype and no genotype x drug interactions at any of the time intervals ($p>0.05$). Tukey's post-tests showed 0.3 mg/kg

SKF-82958 increased distance moved compared to saline at the 0-30 min time point in NT+/+ ($p<0.05$) but not NT-/- mice ($p>0.05$) indicating a lack of hyperlocomotor response to SKF-82958 in NT-/- mice at this dose.

For the 1 mg/kg SKF-82958 (Fig. 2-4b), there was a significant effect of drug at the 0-30 min ($F(1,15)=24.109$, $p<0.001$) and 30-60 min ($F(1,15)=8.227$, $p<0.05$) time intervals, and no significant effects at the 60-90 min time interval. There were no significant effects of genotype and no genotype x drug interaction at any of the time intervals ($p>0.05$). Post-tests showed 1 mg/kg SKF-82958 significantly increased distance moved compared to saline at the 0-30 min time point in both NT+/+ and NT-/- mice ($p<0.01$). 1 mg/kg SKF-82958 increased distance moved at the 30-60 min in NT-/- mice ($p<0.05$) and NT+/+ mice, although, the effect in NT+/+ mice did not reach significance ($p=0.125$). These results indicate 1 mg/kg SKF-82958 produced hyperlocomotion similarly in both NT+/+ and NT-/- mice.

For the 0.1 mg/kg quinpirole (Fig 2-4c), there was a significant effect of drug at the 0-30 min ($F(1,16)=69.87$, $p<0.001$) and 30-60 min time intervals ($F(1,16)=7.802$, $p<0.05$) but not at the 60-90 min time interval ($p>0.05$). There was no significant effect of genotype and no genotype x drug interactions at any of the time intervals ($p>0.05$). Tukey's post-tests indicated 0.1 mg/kg quinpirole decreased distance moved at the 0-30 min interval compared to saline in both genotypes ($p<0.001$). At the 30-60 min time interval, 0.1 mg/kg quinpirole significantly decreased distance moved in the NT+/+ group ($p<0.05$) but not the NT-/- group ($p>0.05$), indicating a slightly decreased duration in hypolocomotor response to quinpirole in NT-/- at this dose. Nonetheless, these results indicate 0.1 mg/kg quinpirole produced hypolocomotion in both NT+/+ and NT-/- mice.

For the 1 mg/kg quinpirole (Fig 2-4d), there was a significant effect of drug at the 60-90 min time interval ($F(1,14)=12.984$, $p<0.01$) and a significant genotype x drug interaction ($F(1,14)=4.763$, $p<0.05$). There were no significant effects at the other two

time intervals (p>0.05). Post-tests showed 1 mg/kg quinpirole significantly decreased distance moved compared to saline at the 60-90 min time point in the NT+/+ mice (p<0.01). The effect did not reach significance in the NT-/- mice (p=0.096). These results indicate a diminished hypolocomotor response to 1 mg/kg quinpirole in the NT-/- mice.

Effects of selective DA receptor agonists on startle amplitude in NT+/+ and NT-/- mice

There were no differences in baseline pulse alone startle amplitude between NT+/+ and NT-/- mice (p>0.05) (Fig. 2-5, inset). The effects of 0.1 mg/kg quinpirole, 1 mg/kg quinpirole, 0.3 mg/kg SKF-82958, and 1 mg/kg SKF-82958 on startle amplitude were analyzed by two-way repeated measures ANOVAs (genotype x drug) (Fig 2-6). All results for experiment #3 are also summarized in Table 3.

For 0.3 mg/kg SKF-82958 (Fig. 2-6a), there was a significant effect of drug ($F(1,16)=15.945$, $p=0.001$) and a genotype x drug interaction ($F(1,16)=7.105$, $p<0.05$). There was no effect of genotype (p>0.05). Tukey's tests showed 0.3 mg/kg SKF-82958 significantly decreased startle amplitude in NT+/+ mice (p<0.001) but did not affect startle amplitude in NT-/- (p>0.05). These results indicate NT knockout blocks the effect of 0.3 mg/kg SKF-82958 on startle amplitude.

For 1 mg/kg SKF-82958 (Fig. 2-6b), there were no significant effects of drug or genotype and no significant genotype x drug interaction (p>0.05). Post-tests showed 1 mg/kg SKF-82958 did not significantly affect startle amplitude in either genotype (p>0.05).

For 0.1 mg/kg quinpirole (Fig. 2-6c), there was a significant effect of drug ($F(1,19)=5.021$,$p<0.05$) and a significant genotype x drug interaction ($F(1,19)=6.413$, $p<0.05$). There was no effect of genotype (p>0.05) on startle amplitude. Post- tests within NT+/+ mice showed 0.1 mg/kg quinpirole administration did not significantly alter startle amplitude compared to saline (p>0.05). In contrast, in the NT-/- mice, 0.1 mg/kg

quinpirole significantly increased startle amplitude compared to saline ($p<0.01$). Thus, in the absence of NT, the effects of 0.1 mg/kg quinpirole on startle amplitude are altered.

For 1 mg/kg quinpirole (Fig 2-6d), there was a significant effect of drug ($F(1,17)=24.359$, $p<0.001$). There were no significant effects of genotype and no genotype x drug interaction. Post-tests showed 1 mg/kg quinpirole significantly decreased startle amplitude in both NT+/+ ($p<0.001$) and NT-/- ($p<0.05$) mice compared to saline controls. These results indicate the effects of 1 mg/kg quinpirole on startle amplitude were similar in both NT+/+ and NT-/- mice.

Effects of selective DA receptor agonists on PPI in NT+/+ and NT-/- mice

There were no differences in baseline PPI between NT+/+ and NT-/- mice ($p>0.05$) (Fig. 2-5). The effects of 0.1 mg/kg quinpirole, 1 mg/kg quinpirole, 0.3 mg/kg SKF-82958, and 1 mg/kg SKF-82958 on PPI were analyzed by two-way repeated measures ANOVAs (genotype x drug). Results for overall PPI (all prepulses combined) are presented in Fig. 2-7 and discussed below. Results for each prepulse are summarized in Table 2. All results for experiment #3 are also summarized in Table 3.

For 0.3 mg/kg SKF-82958 (Fig. 2-7a), there was a significant effect of drug ($F(1,16)=16.975$, $p<0.001$) and no significant effect of genotype ($p>0.05$). A genotype x drug interaction approached significance ($F(1,16)=3.492$, $p=0.080$). Post-tests showed 0.3 mg/kg SKF-82958 significantly decreased PPI in NT+/+ mice ($p<0.01$), but did not significantly decrease PPI in NT-/- mice ($p>0.05$). Thus, in the absence of NT, the disruptive effect of SKF-82958 on PPI at this dose is blocked.

For 1 mg/kg SKF-82958 (Fig. 2-7b), there was a significant effect of drug ($F(1,17)=15.254$, $p=0.001$). There was no significant effect of genotype and no genotype x drug interaction ($p>0.05$). Post-tests showed 1 mg/kg SKF-82958 significantly decreased PPI in both NT+/+ ($p<0.01$) and NT-/- ($p<0.05$) mice.

For 0.1 quinpirole (Fig 2-7c), there was a significant effect of drug ($F(1,19)=6.757$, $p<0.05$). There was no significant effect of genotype and no genotype x drug interaction ($p>0.05$). Post-tests showed 0.1 mg/kg quinpirole did not significantly alter PPI in NT+/+ mice. In contrast, quinpirole increased PPI in NT-/- mice, suggesting that D2 function may be altered in the absence of NT.

For 1 mg/kg quinpirole (Fig 2-7d), the effect of drug approached significance ($F(1,17)=4.162$, $p=0.057$). There was no significant effect of genotype and no genotype x drug interaction ($p>0.05$). Post-tests showed 1 mg/kg quinpirole did not significantly alter PPI in NT+/+ or NT-/- mice.

2.5 DISCUSSION

Locomotor response to amphetamine

These studies sought to investigate the role of NT in the locomotor effects of DA agonists and to examine DA system functioning in the absence of NT. To do this, the consequences of NT gene knockout on the behavioral effects of direct and indirect DA agonists were tested. The first study examined the effects of NT knockout on the acute locomotor-stimulating effects of amphetamine and on sensitization to the locomotor effects of amphetamine (Fig. 2-2). Baseline locomotor activity in NT-/- mice did not differ from locomotion in NT+/+, indicating NT does not play a role in regulating baseline locomotion (Fig. 2-2a). At the lower dose of amphetamine tested (1 mg/kg), both NT+/+ and NT-/- mice showed hyperlocomotor responses to amphetamine. NT-/- mice showed reduced sensitization to the locomotor effects of amphetamine compared to NT+/+ controls, indicating NT plays an important role in moderating amphetamine sensitization. These results concur with previous studies utilizing NTR antagonists that showed blocking NT neurotransmission attenuated amphetamine sensitization (Costa *et al*, 2007; Costa *et al*, 2001; Panayi *et al*, 2002).

In contrast, at the higher dose of amphetamine tested (2 mg/kg), NT-/- mice showed a lack of hyperlocomotor response compared to NT+/+ mice, but both genotypes sensitized to the locomotor effects of amphetamine (Fig. 2-2b). While it is possible that NT-/- mice showed a lack of behavioral response to amphetamine at this dose, another possibility could be that NT-/- mice were showing an exaggerated response to amphetamine. In rodents, low doses of amphetamine produce hyperlocomotion while higher doses result in hypolocomotion due to increased stereotypies (Costall and Naylor, 1974). Thus, it is possible that the lack of hyperlocomotor response in NT-/- mice to amphetamine at the 2 mg/kg dose was due to increased stereotypies. Given that previous studies have shown NT administration has a protective effect against the disruptive behavioral effects of amphetamine (Boules *et al*, 2001; Feifel *et al*, 2008; Feifel *et al*, 1999b), it is possible that mice lacking NT may be supersensitive to the disruptive locomotor effects of amphetamine. To address this possibility, a follow-up experiment investigated stereotyped behaviors in NT+/+ and NT-/- mice after administration of 2 mg/kg amphetamine. NT+/+ and NT-/- mice showed no differences in stereotypies (grooming, rearing, and sniffing) at this dose of amphetamine, and none of the more intense stereotypies (*e.g.*, licking, gnawing) were observed (Fig. 2-3). Thus, it can be concluded that the reason for decreased hyperlocomotor response to amphetamine in NT-/- mice at this dose was not due to increased stereotypies, but was actually due to a diminished hyperlocomotor response to amphetamine.

The results from Experiments #1 and #2 demonstrate that NT knockout attenuates both the acute hyperlocomotor response to amphetamine and locomotor sensitization to repeated amphetamine. Thus, NT plays a moderating role in both the acute locomotor response to amphetamine and amphetamine sensitization. In addition, these studies suggest altered DA system functioning in NT-/- mice, which was further investigated in Experiment #3. NT knockout did not affect amphetamine-induced stereotyped behaviors

at the 2 mg/kg dose, suggesting NT does not play a role in moderating amphetamine-induced stereotypies. However, few stereotypies were observed in the mice at this dose, so future studies might test higher doses of amphetamine in NT+/+ and NT-/- to ascertain whether NT regulates amphetamine-induced stereotypies.

These results agree in part with previous studies utilizing NTR antagonists, which show NTR blockade, like NT gene knockout, attenuates amphetamine sensitization (Costa *et al*, 2007; Costa *et al*, 2001; Panayi *et al*, 2002). However, previous studies show pharmacological NTR blockade does not affect acute amphetamine locomotor response (Cáceda *et al*, 2012; Casti *et al*, 2004). In contrast, NT gene knockout attenuates the acute hyperlocomotor response to 2 mg/kg amphetamine. The discrepancy between our results and studies utilizing NTR antagonists could be due to fundamental differences in the methods utilized. NT gene knockout is a more profound developmental method for blocking NT neurotransmission and when compared to pharmacological NTR antagonism, NT gene knockout might be expected to produce greater deficits. In accordance with this theory, NTR antagonists do not affect baseline PPI in rats (Cáceda *et al*, 2012), but previous studies in our lab show NT gene knockout decreases baseline PPI in mice (Kinkead *et al*, 2005). Taken together, these studies suggest that NT gene knockout may result in developmental alterations in the mesolimbic DA system that could potentially alter behavioral response to amphetamine. This possibility is investigated in the studies presented in Chapter 3.

Conclusions and clinical implications of amphetamine studies

From these studies it can be concluded that endogenous NT plays a role in facilitating both amphetamine sensitization and the acute locomotor response to amphetamine. Repeated use of amphetamine in humans results in the development of psychotic symptoms indistinguishable from those observed in people with schizophrenia. For this reason, the amphetamine sensitization paradigm is sometimes used as an

animal model of psychosis (Peleg-Raibstein *et al*, 2008; Robinson *et al*, 1986). Thus, the results from our studies implicate NT in the neurobiology of psychiatric disorders such as schizophrenia. This implication will be further discussed in Chapter 5.

The amphetamine sensitization paradigm is also a model for the drug-induced plasticity that occurs during the development of psychostimulant addiction. Our studies showed NT gene knockout reduced amphetamine sensitization. Thus, our results implicate NT in the neurobiology of amphetamine addiction. Although clinical data on NT neurotransmission in drug users is lacking, several animal studies show NT is involved in the effects of psychostimulants (for review see St-Gelais *et al*, (2006), Cáceda *et al*, (2006), and Introduction). As reviewed in the Introduction in Chapter 1, systemic amphetamine and cocaine administration increases NT expression and release in the striatum. In addition, centrally administered NT produces some rewarding effects, such as enhancing intracranial self-stimulation (for review see Cáceda et al. 2006). Taken together, these studies suggest NT might be involved in psychostimulant addiction, although more studies are warranted. Future studies might investigate the role of NT in the rewarding effects of psychostimulants. For example, the effects of NT knockout on psychostimulant conditioned place preference or psychostimulant self-administration might be examined.

Behavioral response to direct DA receptor agonists

The effects of amphetamine, an indirect DA receptor agonist, on PPI (Kinkead *et al*, 2005) and locomotion (above studies) are diminished in NT-/- mice. In order to further probe the underlying mechanisms of their altered response to amphetamine, the effects of direct D1- and D2-family agonists on locomotion and PPI in NT-/- mice were investigated. The results of these studies are summarized in Table 2-3.

Baseline startle amplitude and PPI

In contrast to previous studies (Kinkead *et al*, 2005), NT-/- mice did not show baseline differences in startle amplitude or PPI compared to NT+/+ controls (Fig. 2-5). This lack of replication may be due to the smaller sample sizes utilized in this study (*n*=8-13/genotype) compared to the sample sizes used previously (*n*=48-52/genotype). PPI is known to vary widely between individual animals, and it is possible that the PPI deficit observed in previous studies may only be apparent with large sample sizes. Notably, in another study in our lab using larger sample sizes (*n*=20/genotype), a PPI deficit was observed in NT-/- compared to NT+/+ mice for trials with a 4 dB prepulse (data not shown). It is also possible that unknown environmental factors in the animals' housing conditions may have contributed to the lack of replication of PPI deficits in this cohort. Lack of replication of previously observed PPI deficits in another mouse model (neuregulin 1 mutant mice) was also noted and was attributed to minor differences in the laboratory environment and animal housing (Karl *et al*, 2011).

Behavioral response to D1-type agonist

SKF-82958, a D1-family agonist, increased locomotion, decreased startle amplitude (at 0.3 mg/kg), and disrupted PPI in NT+/+ mice as was expected from previous studies (Frau *et al*, 2012). The effects of SKF-82958 on locomotor behavior (Fig. 2-4, Table 2-1), startle amplitude (Fig. 2-6), and PPI (Fig. 2-7, Table 2-2) were dose-dependently diminished in NT-/- mice compared to NT+/+ mice (see Table 2-3 for summary). Significant behavioral effects of the low dose of SKF-82958 (0.3 mg/kg) were absent in NT-/- compared to NT+/+. Our results indicate D1-type receptor function, as it pertains to locomotor and PPI disruption, is blunted in the absence of NT. These results concur with one previous study utilizing an NTR antagonist that showed blocking NT neurotransmission decreased the behavioral effects of another selective D1 receptor agonist (Poncelet *et al*, 1994). These results also parallel the results from experiment #1 showing a blunted behavioral response to amphetamine in NT-/- mice. It is possible that

the decreased response to amphetamine in NT-/- mice might also be due to diminished D1-type function. In contrast, the behavioral effects of the higher dose of SKF-82958 (1 mg/kg) on locomotor activity and PPI in NT-/- mice were similar to those in NT+/+ mice. The lack of prominent effect of NT knockout on behavioral response to 1 mg/kg SKF-82958 may be due to the overwhelming effect of the agonist at this high dose. Thus, lack of NT diminishes the disruptive behavioral effects of the D1 agonist, but this can be compensated for with higher doses. Nonetheless, these results indicate that D1-type function, as it pertains to locomotor and PPI disruption, is blunted in the absence of NT.

Locomotor response to D2-type agonist

Quinpirole, a D2-family agonist, decreased locomotion at both doses in NT+/+ mice (Fig. 2-4, Table 2-1). Some studies report higher doses of quinpirole increase locomotor activity in rats (Eilam and Szechtman, 1989; Eilam *et al*, 1992; Horvitz *et al*, 2001) and mice (Jung *et al*, 2011; Luque-Rojas *et al*, 2012), while others report all doses of quinpirole inhibit locomotion in mice (Halberda *et al*, 1997). In agreement with Halberda *et al*, (1997), our results show both high and low doses of quinpirole significantly decreased locomotion in NT+/+ mice. Hypolocomotor response to 0.1 mg/kg quinpirole was similar in NT-/- compared to NT+/+ mice. However, at the 1 mg/kg dose, NT-/- mice did not show a significant hypolocomotor response to quinpirole. These studies suggest altered D2-like receptor function in NT-/- mice.

Previous studies suggest the mechanism of quinpirole's inhibitory effect on locomotion is stimulation of midbrain D2 autoreceptors (D2S isoform) and a subsequent decrease in DA release into the striatum (Imperato *et al*, 1988; Wang *et al*, 2000). Larger doses of quinpirole also stimulate post-synaptic striatal D2 receptors and may produce increased locomotion (Imperato *et al*, 1988). As only decreases in locomotion (no increases) in response to both doses of quinpirole were observed in our studies, the observed hypolocomotor effects of quinpirole might be due primarily to stimulation of D2

autoreceptors. As NT-/- mice showed a lack of hypolocomotor response to quinpirole at the 1 mg/kg dose, it might be speculated that D2 autoreceptor function in NT-/- is diminished. This result was unexpected as the effects of quinpirole were expected to be exacerbated in the absence of NT given the inhibitory actions of NT on the D2 receptor. Previous studies show central injection of NT attenuates the effects of quinpirole stimulation of midbrain D2 autoreceptors on DA cell firing (Shi *et al*, 1990; Shi *et al*, 1991). Therefore it was predicted that the D2 autoreceptor-mediated inhibition of locomotion by quinpirole might be increased in NT-/- mice. However, quinpirole-mediated inhibition of locomotion was in fact absent in NT-/- mice. Whether the lack of response to quinpirole in NT-/- mice is due to diminished function in D2 autoreceptors needs to be determined in follow-up experiments. Future experiments utilizing central injection of quinpirole and drugs selective for D2 receptor subtypes in NT+/+ and NT-/- mice are warranted to pinpoint the location and specific receptor subtypes involved in the observed behavioral alterations in NT-/- mice.

Effects of D2-type agonist on PPI and startle amplitude

In NT+/+ mice, the lower dose of quinpirole (0.1 mg/kg) did not significantly affect pulse alone startle amplitude (Fig. 2-6) or PPI (Fig. 2-7, Table 2-2). These results concur with previous studies showing quinpirole does not disrupt PPI in C57BL/6J mice (Ralph-Williams *et al*, 2003; Ralph-Williams *et al*, 2002b) as it does in rats (Peng *et al*, 1990; Wan *et al*, 1994). In contrast, the low dose of quinpirole increased pulse alone startle amplitude (Fig. 2-6) and enhanced PPI (Fig. 2-7) in NT-/- mice. As mentioned above, the effects of quinpirole in NT-/- mice were expected to be exacerbated given the known inhibitory actions of NT on D2 receptor function (Shi *et al*, 1990; Shi *et al*, 1991). As quinpirole (0.1 mg/kg) had no effect on startle amplitude and PPI in wildtype C57BL/6J mice, it was predicted that quinpirole might decrease startle amplitude and disrupt PPI in mice lacking NT. Surprisingly, quinpirole (0.1 mg/kg) had the opposite

effect of increasing startle amplitude and enhancing PPI. Interestingly, intra-accumbal injection of quinpirole in mice enhances PPI (Mohr *et al*, 2007), an effect that contrasts with systemic administration of quinpirole, which has no effect on PPI in mice (Ralph-Williams *et al*, 2003; Ralph-Williams *et al*, 2002b). As systemic D2 receptor agonism in NT-/- mice had effects similar to intra-accumbal D2 receptor agonism in wildtype mice (increased PPI), it might be hypothesized that NT-/- mice may have alterations in D2-like receptor expression or function in the NAcc that may bias the behavioral effects of systemic D2 receptor agonism. This possibility is investigated in the studies presented in Chapter 3. However, as our studies utilized systemic injections, future studies utilizing accumbal injection of quinpirole in NT+/+ and NT-/- mice are in order to determine if the observed effects of quinpirole in NT-/- are due to D2-like agonism in the NAcc.

In some, NT-/- mice showed a blunted locomotor response to quinpirole compared to NT+/+ mice, whereas NT-/- mice showed increased effects of quinpirole on startle response and PPI compared to NT+/+ mice. Thus, it is unclear if D2 function in NT-/- mice is blunted or enhanced. Future experiments could address this issue by testing D2 function more directly by investigating the effects of *in vivo* central injection of quinpirole and/or receptor subtype-specific ligands on changes in DA cell activity and DA efflux. Follow-up experiments might also probe D2 function in NT-/- mice by measuring possible changes in receptor-stimulated G protein coupling or cAMP cascade. Nevertheless, from these studies, it can be concluded that D2-like function is altered in NT-/- mice.

General conclusions and future directions

In sum, our studies show NT modulates the behavioral effects of both direct and indirect DA receptor agonists on locomotor activity and PPI. NT plays a role in both the acute locomotor response to amphetamine and amphetamine sensitization. In addition, both D1-like and D2-like function, as it pertains to the disruption of locomotor behavior,

startle response, and PPI, are altered as a consequence of NT gene knockout. D1-type function is blunted in the absence of NT. D2-type function, perhaps D2 autoreceptor function, is also altered as it pertains to these behaviors. These results may explain the underlying mechanisms for previously obtained findings in NT-/- mice. NT-/- mice previously showed deficits in PPI (Kinkead *et al*, 2005). Altered D1 function in NT-/- mice might explain the previously observed baseline deficits in NT-/- mice as D1 is essential for regulating PPI in mice (Ralph-Williams *et al*, 2002b). In Experiment #1, NT-/- mice also showed decreased acute locomotor response to amphetamine and reduced amphetamine sensitization. Blunted D1 receptor function may explain the observed diminished locomotor response to amphetamine as the D1 receptor is necessary for both acute locomotor response to amphetamine and amphetamine sensitization (O'Neill *et al*, 1999; Vezina, 1996). These explanations are further discussed in Chapter 5.

As these studies utilized systemic injections of agonists, we cannot precisely determine where the loss of function is in NT-/- mice. Given the rich literature on the mechanisms of DA receptor agonist disruption of locomotor behavior and PPI, however, we can theorize that D1-like and D2-like receptor function within the mesolimbic and perhaps nigrostriatal DA systems might be altered in NT-/- mice compared to NT+/+ mice. Future studies might utilize *in vivo* injection of DA receptor agonists into the NAcc, CP, and VTA to pinpoint region specificity. Finally, it is possible that the altered response to amphetamine and direct DA agonists observed in NT-/- mice in these studies might be caused by developmental alterations in the mesolimbic DA system caused by knockout of the NT gene. Chapter 3 investigates this possibility by characterizing cortical and striatal DAergic tone in mice lacking NT.

FIGURES

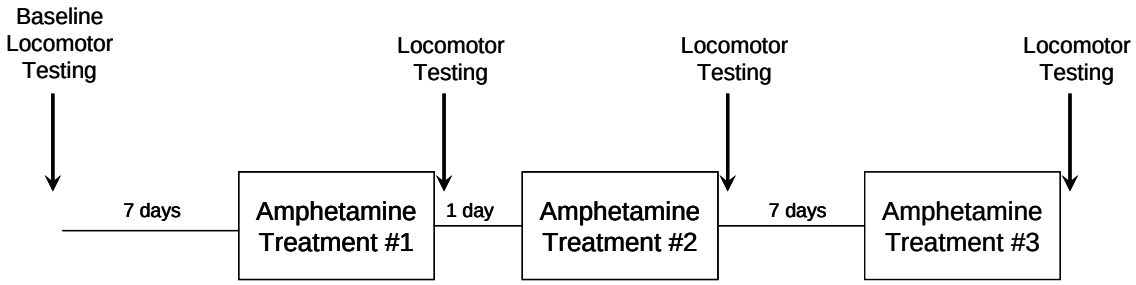

Fig. 2-1. Experimental design for experiment #1, the effects of amphetamine on locomotor behavior in NT+/+ and NT-/- mice.

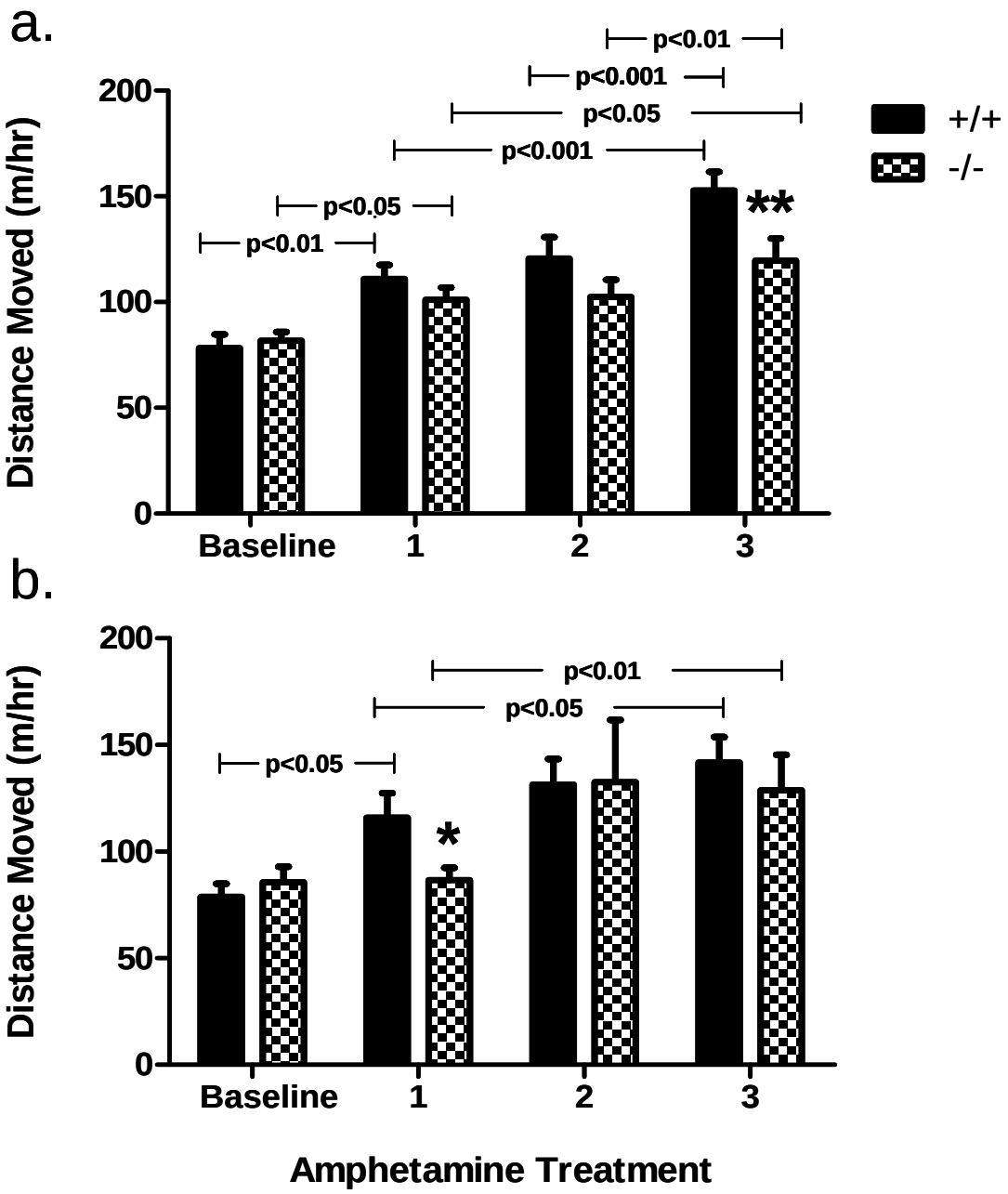

Fig 2-2. Effects of 1 mg/kg (a) and 2 mg/kg (b) acute and subchronic amphetamine on locomotor behavior in NT+/+ and NT-/- mice. Data are expressed as mean distance moved (m/hr) ± S.E.M. The x axis labels indicate treatment number. NT-/- showed blunted acute locomotor response to amphetamine (amphetamine 1) at the 2 mg/kg dose (b) and did not sensitize to the hyperlocomotor effects of amphetamine

(amphetamine 3) at the 1 mg/kg dose (a). *p<0.05,**p<0.01, NT-/- compared to NT+/+ within the same treatment session.

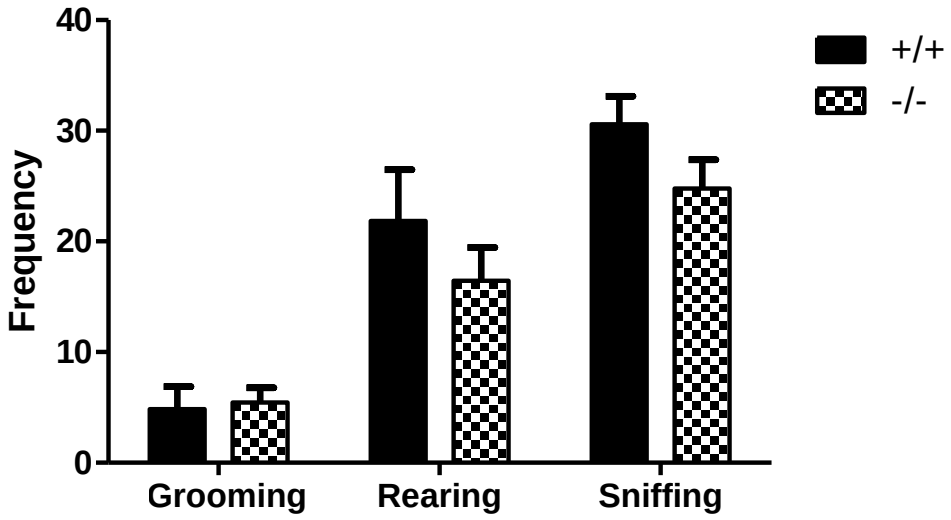

Fig. 2-3. The effects of 2 mg/kg amphetamine on stereotyped behavior in NT+/+ and NT-/- mice. There were no significant differences (p>0.05) in stereotyped behaviors between NT+/+ and NT-/- mice.

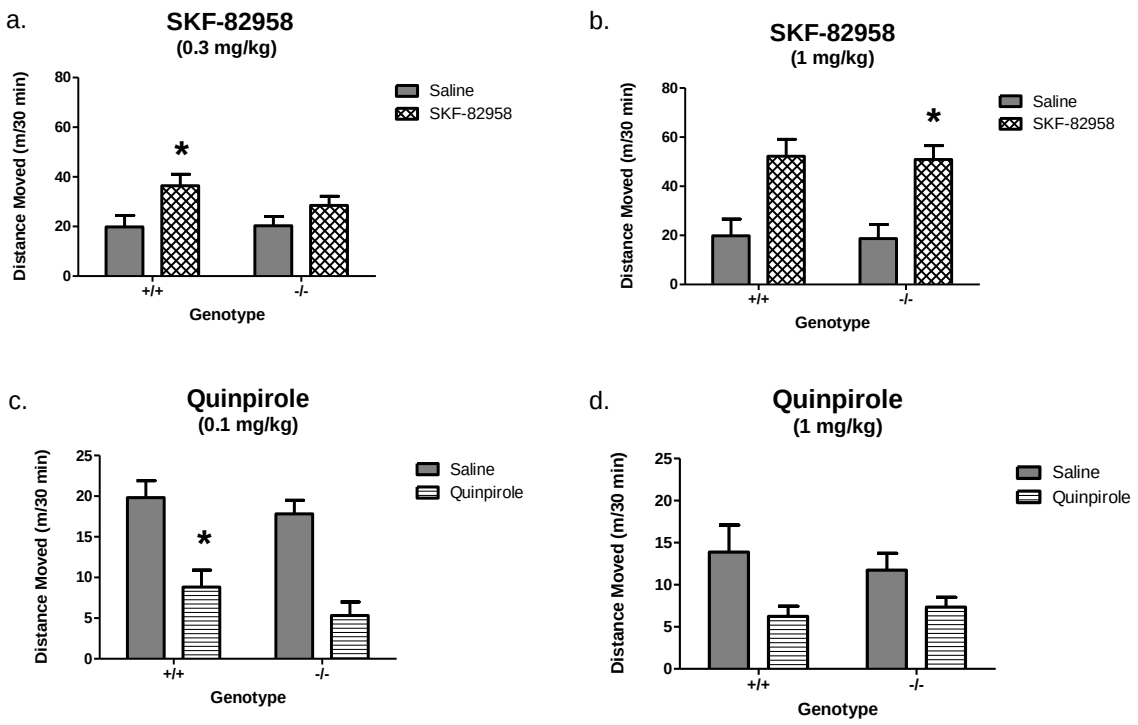

Fig 2-4. Effects of SKF-82958 and quinpirole on distance moved (m) in NT+/+ and NT-/- mice. Data presented in (a-c), are from the 0-30 min time window, while data presented in (d) are from the 60-90 min time window. Data are expressed as mean distance moved (m/30 min) ± S.E.M. *p<0.05, drug treatment compared to saline control within genotype.

	0-30 min		30-60 min		60-90 min	
Drug	**+/+**	**-/-**	**+/+**	**-/-**	**+/+**	**-/-**
Saline	19.816 ± 2.816	17.721 ± 1.743	12.337 ± 3.551	10.700 ± 1.885	13.883 ± 3.213	11.231 ± 1.913
0.1 mg/kg Quinpirole	**8.820 ± 1.977***	**5.330 ± 0.816***	**5.883 ± 1.375***	7.110 ± 1.191	10.289 ± 1.845	9.211 ± 1.573
1 mg/kg Quinpirole	18.500 ± 3.828	19.523 ± 4.658	5.539 ± 1.885	13.500 ± 5.016	**5.785 ± 1.412***	7.358 ± 1.293
0.3 SKF-82958	**36.439 ± 5.418***	28.491 ± 4.736	14.633 ± 3.305	10.192 ± 1.064	9.966 ± 1.629	7.151 ± 1.890
1 mg/kg SKF-82958	**52.144 ± 7.309***	**50.870 ± 8.490***	26.539 ± 6.076	**30.609 ± 7.607***	10.646 ± 3.087	14.073 ± 3.370

Table 2-1. Effects of quinpirole and SKF-82958 on distance moved (m) in NT+/+ and NT-/- mice. Data are expressed as mean distance moved (m) ± S.E.M. *p<0.05, drug treatment compared to saline control within genotype.

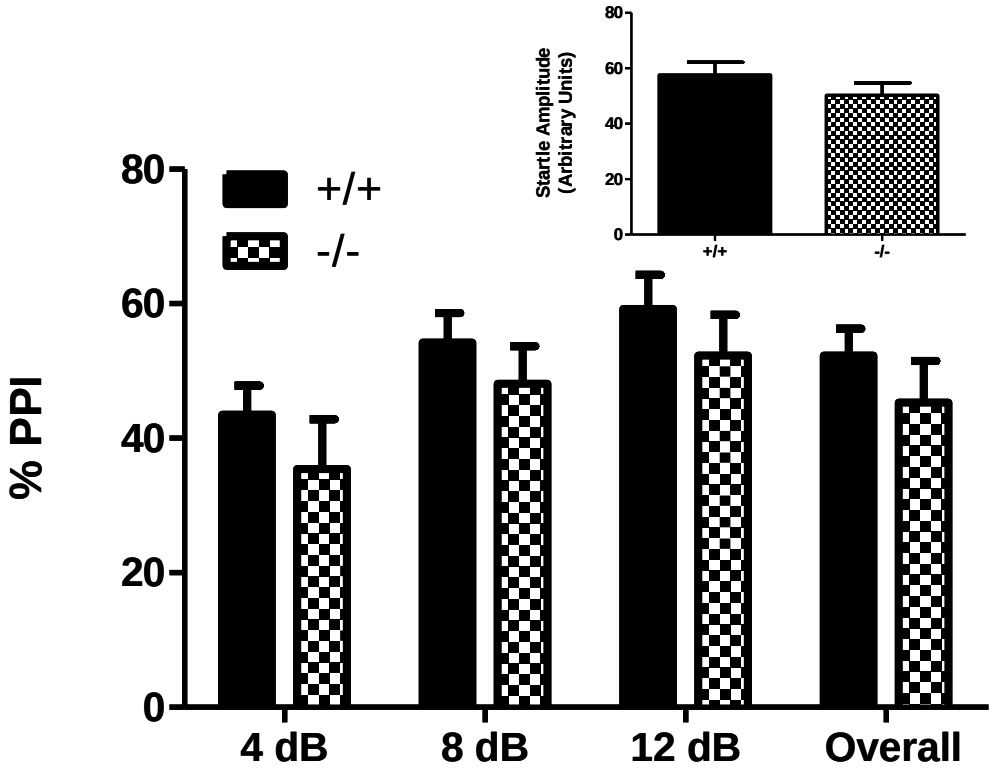

Fig 2-5. Baseline startle amplitude (inset) and PPI in NT+/+ and NT-/- mice. Data are presented as mean startle amplitude ± S.E.M. and mean PPI ± S.E.M. PPI are presented by each prepulse and as all prepulses combined (overall).

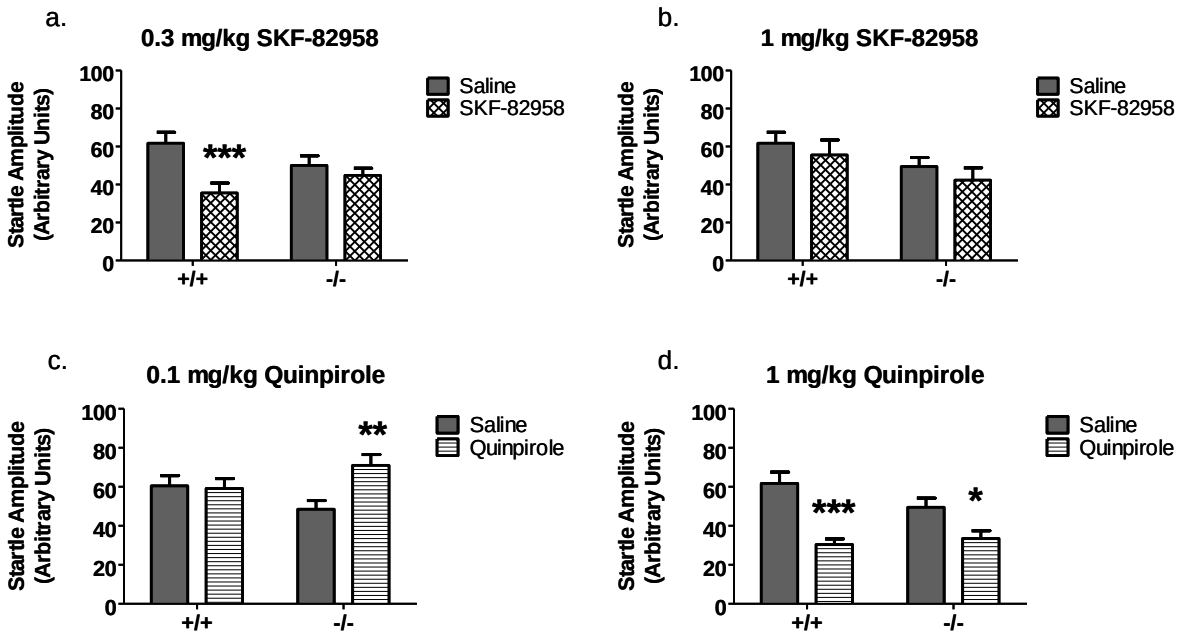

Fig. 2-6. Effects of SKF-82958 and quinpirole on startle amplitude in NT+/+ and NT-/- mice. Data are presented as mean startle amplitude ± S.E.M. *p<0.05, **p<0.01, drug treatment compared to saline control within genotype, ***p<0.001).

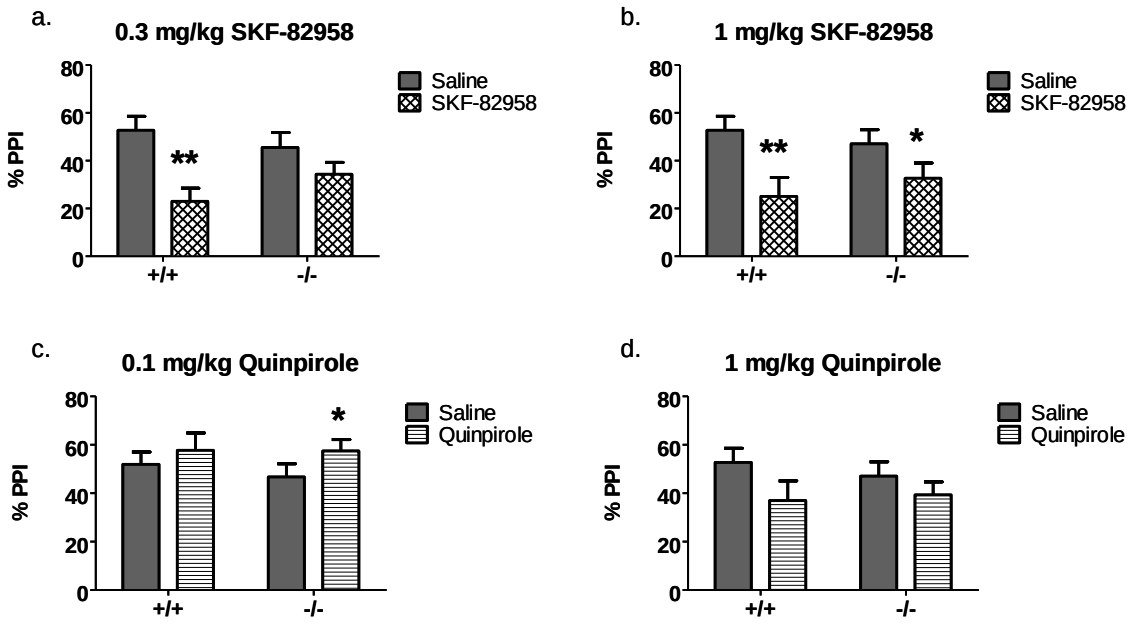

Fig. 2-7. Effects of SKF-82958 and quinpirole on overall PPI in NT+/+ and NT-/- mice. Data are presented as mean % PPI ± S.E.M. *p<0.05, **p<0.01, baseline compared to drug treatment within genotype.

	4 dB		8 dB		12 dB		Overall	
Drug	**+/+**	**-/-**	**+/+**	**-/-**	**+/+**	**-/-**	**+/+**	**-/-**
Saline	45.0 ± 9.5	38.5 ± 7.3	54.2 ± 7.4	49.0 ± 5.7	58.8 ± 8.1	53.6 ± 6.2	52.7 ± 7.7	47.0 ± 5.9
0.1 mg/kg Quinpirole	45.9 ± 7.2	48.3 ± 5.6	61.8 ± 6.7	**61.8 ± 5.3***	65.4 ± 6.4	**62.2 ± 5.1***	57.7 ± 6.4	**57.4 ± 5.0***
1 mg/kg Quinpirole	25.4 ± 8.8	33.4 ± 6.8	40.8 ± 6.6	38.6 ± 5.0	44.8 ± 7.8	45.9 ± 5.9	37.0 ± 7.3	39.3 ± 5.6
0.3 SKF-82958	**16.6 ± 8.2****	26.2 ± 6.5	**23.8 ± 6.6****	35.1 ± 5.3	**28.4 ± 6.9*****	41.6 ± 5.5	**22.9 ± 6.6****	34.3 ± 5.3
1 mg/kg SKF-82958	**15.4 ± 9.5***	27.7 ± 7.3	**24.5 ± 7.4****	**32.6 ± 5.7***	**35.3 ± 8.1***	**37.8 ± 6.2***	**25.0 ± 7.7****	**32.7 ± 5.9***

Table 2-2. Effects of quinpirole and SKF-82958 on PPI in NT+/+ and NT-/- mice. Data are presented as mean % PPI ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 baseline compared to drug treatment within genotype.

	Locomotor Activity		Startle Amplitude		PPI	
Drug	+/+	-/-	+/+	-/-	+/+	-/-
0.3 mg/kg SKF-82958	Increased	No effect	Decreased	No Effect	Decreased	No Effect
1 mg/kg SKF-82958	Increased	Increased	No Effect	No Effect	Decreased	Decreased
0.1 mg/kg Quinpirole	Decreased	Decreased	No Effect	Enhanced	No effect	Enhanced
1 mg/kg Quinpirole	Decreased	No effect	Decreased	Decreased	No effect	No Effect

Table 2-3. Effects of D1-type and D2-type receptor agonists on behaviors in NT-/- mice compared to NT+/+ mice.

3. THE CONSEQUENCES OF NEUROTENSIN DEFICIENCY ON DOPAMINERGIC TONE

3.1 ABSTRACT

NT is a modulator of DA neurotransmission. NT gene knockout results in altered behavioral effects of DA receptor agonists on locomotor behavior and PPI, behaviors regulated by the nigrostriatal and mesocorticolimbic DA systems. These studies suggest altered function in the nigrostriatal and mesocorticolimbic DA systems in NT-/- mice. Although disrupting NT neurotransmission by NT gene knockout is known to result in these behavioral alterations, it is unknown whether NT gene knockout produces developmental changes in these DA systems. These experiments investigated the possibility of developmental alterations in DAergic tone in DA system terminal regions, the NAcc, frontal cortex (FCTX), and caudate putamen (CP), in NT-/- mice compared to NT+/+ mice. NT-/- mice did not differ from NT+/+ mice in concentrations of DA or its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in any of the brain regions examined. However, NT-/- mice showed significantly increased D1 receptor, D2 receptor, and DAT mRNA in the CP compared to NT+/+ controls. In addition, D1 receptor expression in both the NAcc and FCTX was found to be correlated with individual differences in PPI in NT+/+ mice. However, these correlations were absent in NT-/- mice, suggesting a decoupling of DA systems and DA-sensitive behavior in the absence of NT. Finally, NT-/- mice also showed elevated D2 receptor binding density in both the CP and NAcc shell compared to NT+/+ mice. The results from this study show that NT deficiency during development permanently alters striatal DA receptor expression and binding, which may explain some of the behavioral alterations in NT-/- mice. In sum, these data support a critical role for the NT system in the development of the DA systems, particularly in the striatal terminal regions.

3.2 INTRODUCTION

Research on the etiology of schizophrenia has shown disrupted DAergic activity in the mesolimbic pathway to be an important underlying factor (Grace, 1991). The 'dopamine hypothesis' postulates that some schizophrenia symptoms are due to hyperactivity of the mesolimbic DA system, as drugs that increase DAergic activity in this system, like amphetamine, produce psychotic behaviors similar to those observed in schizophrenia (Lieberman *et al*, 1987; Meltzer *et al*, 1976; van Rossum, 1966). However, this causal explanation has proven to be too simple, and the issue is far from being completely understood. Clinical studies have failed to find increases in central DA or DA metabolites. In fact, there is evidence that DA turnover might actually be depressed in people with schizophrenia, and inhibitory striatal D2 receptors are often found to be increased (Grace, 1991; Seeman, 1987; Seeman *et al*, 2000; Wong *et al*, 1997). In addition, other neurotransmitters besides DA have also been implicated in the neuropathology of schizophrenia, and NT is one of them. Several clinical studies implicate NT in the neurobiology of schizophrenia. Decreased concentrations of NT are found in the cerebrospinal fluid (CSF) of a subset of schizophrenic patients (Breslin *et al*, 1994; Lindström *et al*, 1988; Nemeroff *et al*, 1989a; Sharma *et al*, 1997), and NT levels normalize following effective treatment with antipsychotic drugs (Garver *et al*, 1991; Widerlöv *et al*, 1982). These studies led to the hypothesis that NT may act as an endogenous antipsychotic drug (Nemeroff, 1980).

Studies utilizing central injection of NT and NTR antagonists have shown NT serves as a potent modulator of the nigrostriatal and mesocorticolimbic DA systems (Binder *et al*, 2001b) (see Chapter 1 for review). In addition, as shown by previous studies (Cáceda *et al*, 2012; Costa *et al*, 2001; Kinkead *et al*, 2005) and in the studies in Chapter 2, the effects of pharmacological manipulation of these DA systems are known

to be dependent on intact NT neurotransmission. Specifically NTR antagonism (Cáceda *et al*, 2012; Costa *et al*, 2001) and deletion of the NT gene (Kinkead *et al*, 2005) alter PPI and locomotor response to DA receptor antagonists and agonists. As previously described, the disruptive effects of DA receptor agonists on PPI and locomotor behavior are known to be regulated by the mesocorticolimbic and nigrostriatal DA systems. The regional immediate-early gene response to pharmacological activation of these DA systems is also altered by blocking NT neurotransmission. Particularly, drug-induced increases in immediate-early gene expression (*c-fos* mRNA and Fos protein*)* in the dorsal striatum produced by haloperidol (a D2 receptor antagonist) are attenuated by NTR antagonism (Binder *et al*, 2004; Fadel *et al*, 2001) and NT gene knockout (Dobner *et al*, 2001). Amphetamine-induced increases in *c-fos* mRNA and Fos protein are also diminished in the medial striatum (Fadel *et al*, 2006), FCTX, and NAcc (Cáceda *et al*, 2012) by NTR antagonism and are reduced in the medial striatum by NT gene knockout (Fadel *et al*, 2006). These studies demonstrate functional changes in both the mesocorticolimbic and nigrostriatal DA systems in the absence of NT.

Although disrupting NT neurotransmission by NT gene knockout is known to result in altered responses to activation of the DA systems, it is unknown whether a deficit in NT produces any developmental changes in these circuits. In light of these previous studies, the experiments detailed in this chapter sought to evaluate DAergic tone in terminal regions in NT-/- mice compared to NT+/+ mice. Specifically, these studies examined DA receptor and DAT gene expression and DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations in the NAcc, CP, and FCTX.

One of the hallmark symptoms of schizophrenia is disrupted sensorimotor gating. Particularly, schizophrenic patients show deficits in PPI, a measure of sensorimotor gating (Kumari *et al*, 2000). NT has been shown to have an essential role in regulating PPI. Intra-accumbal NT injection increases baseline PPI and blocks amphetamine

disruption of PPI in rats (Feifel *et al*, 1997). In addition, overexpression of NTRs in the NAcc blocks the disruptive effect of amphetamine on PPI in rats (Cáceda *et al*, 2005). Finally, disruptions in the NT system cause disruptions in PPI; specifically, knockout of the NT gene in mice disrupts PPI (Kinkead *et al*, 2005). In light of the strong experimental evidence for the link between NT activity, DA activity, and PPI, physiological data on DA targets in these experiments were correlated with PPI data in the presence or absence of NT. Specifically, DA concentrations and expression levels of DA receptors were correlated with PPI data from each animal to determine the relationship of DAergic tone in terminal regions with PPI values in both NT+/+ and NT-/- mice.

3.3 METHODS

Animals

NT knockout mice were generated as previously described in P.R. Dobner *et al* (2001) and in Chapter 2. Only male mice (60 days of age and older) from the lab's NT knockout breeding colony were used.

Genotyping

Animals were genotyped using the same procedures described in Chapter 2.

Startle Response and PPI Testing

All mice used for tissue in these studies first underwent startle testing in San Diego Instruments (San Diego, CA) startle chambers as previously described in E.B. Binder et al. (2001a) and in Chapter 2.

Experiment 1

HPLC

Concentrations of DA and its metabolite DOPAC were assayed in NT+/+ (n=6-8) and NT-/- (n=8-12) mice by high pressure liquid chromatography (HPLC). Mice were euthanized by decapitation and brains were collected and quickly frozen on dry ice. Brains were later dissected according to The Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos, 1997). NAcc, caudate putamen (CP), and frontal cortex (FCTX) regions were collected. Samples of mouse brains were prepared by adding 200 µl of ice-cold 0.1N perchloric acid containing 0.01% sodium metabisulfite and 25 ng/ml internal standard 3,4-dihydroxybenzylamine hydrobromide to the tissue. Samples were then homogenized and centrifuged at 15 000 x g. for 10 min at 4ºC. The supernatant was injected at a constant flow rate of 1 mL/min onto an Ultrasphere ODS 250 x 4.6 mm column, 5 µm (Beckman Coulter, Fullerton, CA) with mobile phase (0.1mM EDTA; 0.35mM sodium octyl sulfate; 0.6% phosphoric acid; 5% acetonitrile at pH 2.7). A coulometric electrochemical array detector (Agilent Technologies, Santa Clara, CA; guard cell set at 600mV and analytical cell at 300 mV) was used to visualize the peaks. The retention time, height, and area of DA and DOPAC peaks were compared with reference standard solutions (Sigma, St. Louis, MO) and quantified by ChemStation chromatography software (Agilent Technologies, Santa Clara, CA). For each sample, DA and DOPAC amounts were normalized to total protein as determined by the Lowry Assay. Concentration values for each animal were correlated with PPI values in linear regression analyses.

Experiment 2

Real Time RT-PCR

mRNA levels of the DAT, D1 receptor, and D2 receptor in NT+/+ (n=8 pairs) and NT-/- (n=8 pairs) mice by real time reverse transcriptase (RT)-PCR. Mice were euthanized by decapitation and brains were collected and quickly frozen on dry ice. Brains were later

dissected according to The Mouse Brain in Stereotaxic Coordinates (Franklin & Paxinos 1997). NAcc, CP, FCTX, and VTA regions were collected and pooled together in pairs from the same genotype matched on overall % PPI values to generate enough tissue for RNA extraction. RNA from these regions of interest were then extracted by the TRIzol method (Invitrogen, Carlsbad, CA) and reverse transcribed with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA).

Before running samples, a mouse endogenous control plate (Applied Biosystems, Foster City, CA) was utilized to determine the ideal endogenous control. The gene showing the least variation in expression between the genotypes was *Polr2a* (gene encoding polymerase (RNA) II (DNA directed) polypeptide A), and it was selected as the endogenous control gene for this experiment.

cDNA was quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc, Pittsburgh, PA). Primers for *Slc6a3* (gene encoding DAT), *Drd1a* (gene encoding D1), *Drd2* (gene encoding D2), and *Ntsr1* (gene encoding NTS1) targets were purchased from Applied Biosystems Assays on Demand (Applied Biosystems, Foster City, CA). RT-PCR was performed on the Applied Biosystems 7900HT system (Applied Biosystems, Foster City, CA). dC_T values were calculated by subtracting the C_T of the target gene from the C_T of the endogenous control gene for each sample. Individual $-dC_T$ values were correlated with PPI values in linear regression analyses. ddC_T values were calculated by subtracting the mean dC_T of NT+/+ from the mean dC_T of NT-/-. Gene expression changes were then assessed with the following formula: Fold change in gene expression = 2^{-ddC_T}.

Experiment 3

Receptor and transporter binding autoradiography

NT+/+ and NT-/- mice (n=8/genotype) were euthanized by decapitation and brains were collected and quickly frozen on dry ice. Brains were sectioned on a cryostat at 25 µm thickness and mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA). Slides were stored at -70ºC until autoradiography, and alternative sections from the same brains were used for separate binding assays. For D1 binding, sections were incubated for 90 min at room temperature in the presence of 4 nM [^{3}H]-SCH23390 (Perkin Elmer, Waltham, MA) and 1 µM mianserin (MP Biomedicals Inc), in order to avoid the binding of [^{3}H]-SCH23390 to 5-HT2 and 5-HT1c receptors. Non-specific binding was determined in the presence of 10 µM unlabeled *cis*-flupenthixol (Santa Cruz Biotechnology). To label D2 receptors, sections were incubated for 60 min in the presence of 4 nM [^{3}H]-raclopride (Perkin Elmer, Waltham, MA) and 10 nM 7-OH-DPAT (Sigma-Aldrich, St. Louis, MO) in order to avoid the binding of [^{3}H]-raclopride to D3 receptors. Non-specific binding was determined in the presence of 10 µM sulpiride (Sigma-Aldrich, St. Louis, MO). Slides were exposed to BAS-5000 phosphoimaging plates (FujiFilm) for 48 hours (D1 binding) or 12 days (D2 binding) along with tritium standards (American Radiolabeled Chemicals, Inc., St. Louis, MO). For DAT binding, sections were incubated for 60 min in 20 pM [^{125}I]-RT1-121 (Perkin Elmer, Waltham, MA). Non-specific binding was determined in the presence of 200 µM unlabeled nomifensine maleate (Sigma-Aldrich, St. Louis, MO).The sections and ^{14}C plastic standards (Amersham Biosciences) were exposed to BioMax MR film (Kodak) for 48 hours.

Image and Film Quantification

For D1 and D2 autoradiography, the BAS-5000 plates (FujiFilm) were developed in a BAS-5000 phosphorimager (FujiFilm) and images were analyzed using MultiGauge software (FujiFilm). Photostimulated luminescence per mm^2 (PSL/ mm^2) was measured for regions of interest bilaterally. PSL/ mm^2 were converted to nanocuries/mg protein with tritium standards (American Radiolabeled Chemicals, Inc., St. Louis, MO). For DAT

autoradiography, BioMax MR films (Kodak) were analyzed by quantitative densitometry using AIS computerized software (AIS, St. Catherines, Ontario, Canada). Optical densities were measured for regions of interest bilaterally, and were converted to nanocuries/mg protein with the ^{14}C standards (Amersham Biosciences).

To determine regions of interest, slide-mounted sections were dyed using Neutral Red and were compared to a mouse atlas (Franklin *et al*, 1997). For all sections, two densitometry measurements were made in the NAcc bilaterally, one at bregma +1.34 mm and one between bregma +1.18 mm and +1.1 mm. Densitometries were sampled in the NAcc core in a rectangular area and in the NAcc shell using the free hand tracing tools in MultiGauge and AIS. The CP was measured bilaterally between bregma +1.18 mm and +0.98 mm. Densitometries were sampled in CP subregions (dorsomedial, dorsolateral, ventromedial, and ventrolateral regions) in rectangular areas and in the total CP using the free hand tracing tools in MultiGauge and AIS. Background was subtracted from regions of interest from adjacent areas lacking specific binding on the same section. Densitometry values for each animal were correlated with PPI values in linear regression analyses.

Statistical Analysis

For these experiments, differences between genotypes for each target from HPLC, gene expression, and autoradiography studies were analyzed by a Student's *t* test. For the gene expression studies, Pearson correlations were calculated between $-dC_T$ values across brain regions and between % PPI and $-dC_T$ values. For the HPLC studies, Pearson correlations were calculated between % PPI and DA and DOPAC concentrations. For the autoradiography studies, Pearson correlations were calculated between % PPI and binding densities. Significance was set at $p<0.05$ for all analyses.

Statistical analyses were performed with GraphPad Prism 3.0 (GraphPad Sortware, San Diego, CA) and SysStat SigmaPlot 12.3 software.

3.4 RESULTS

DA and DOPAC concentrations in NT+/+ and NT-/- mice

Differences in DA and DOPAC concentrations in the NAcc, CP, and FCTX in NT+/+ and NT-/- mice were examined by HPLC. There were no significant differences in DA, DOPAC, or DOPAC/DA ratio between NT+/+ and NT-/- mice in any of the brain regions examined (Table 3-1). There was also no difference in DA and DOPAC concentrations in the NAcc compared to the FCTX (NAcc/FCTX ratio), but there was a significant increase in DOPAC/DA ratio in the NT-/- mice compared to the NT+/+ mice when these brain regions were compared ($p<0.05$) (Table 3-1). This result suggests there may be an increase in DA utilization in the NAcc relative to the FCTX in NT-/- mice compared to NT+/+ mice.

Gene Expression in NT+/+ and NT-/- mice

Differences in mRNA levels of the D1 receptor, D2 receptor, and the DAT were examined in NT+/+ and NT-/- mice by real time RT-PCR (Table 3-2). NT-/- mice had significantly increased D1, D2, and DAT mRNA compared to NT+/+ mice in the CP. NT-/- mice showed a trend for increased DAT mRNA in the FCTX compared to NT+/+ mice, but this difference did not reach significance ($p=0.07$). There were no significant differences in mRNA levels of any of the targets in the NAcc between genotypes.

In addition, D1 and D2 mRNA levels ($-dC_T$ values) were analyzed for regional correlation between the FCTX and NAcc. Receptor expression in the FCTX was not correlated with its expression in the NAcc in either genotype ($p>0.05$).

DA receptor and transporter binding in NT+/+ and NT-/- mice

Differences in striatal DAT, D1-like, and D2-like binding densities in NT+/+ and NT-/- mice were examined (Fig. 3-1). There were no significant differences in DAT binding (Fig. 3-2a) or D1-like (Fig. 3-2b) binding densities in the NAcc or CP between the genotypes ($p>0.05$). However, NT-/- mice had significantly increased D2-like binding in the CP (at Bregma 1.18 mm-0.98 mm) and NAcc (at Bregma 1.18-1.1 mm but not at Bregma 1.34 mm). Specifically, NT-/- mice showed increased D2-like densities in the NAcc shell, dorsomedial CP, and total CP regions compared to NT+/+ mice ($p<0.05$) (Fig. 3-2c). NT-/- mice showed trends for increased D2-like binding in the NAcc core ($p=0.07$), dorsolateral CP ($p=0.055$), and ventromedial CP ($p=0.07$) which approached significance. NT-/- mice also showed a significantly increased D2/D1 ratio in the NAcc shell and in the dorsomedial, dorsolateral, ventrolateral, and total CP regions ($p<0.05$) (Fig. 3-2d). NT-/- mice showed trends for increased D2/D1 ratio in the NAcc core ($p=0.07$) and ventromedial CP ($p=0.054$).

Startle response and PPI in NT+/+ and NT-/- mice

Pulse alone startle amplitude and PPI were measured in all animals before tissue was collected for the HPLC experiment (Experiment 1), RT-PCR experiment (Experiment 2) and the autoradiography experiment (Experiment 3). For startle amplitude, t-tests showed no differences between NT+/+ and NT-/- mice (cohort 1: $t(16)=1.050$, $p>0.05$; cohort 2: $t(14)=0.316$, $p>0.05$;cohort 3: $t(14)=0.041$, $p>0.05$). In both experimental cohorts, there were no significant differences in PPI between NT+/+ and NT-/- in PPI at any of the prepulse intensities ($p>0.05$). Likewise, there was no difference in overall % PPI (all prepulses combined) between NT+/+ and NT-/- mice (cohort 1: $t(16)=0.415$, $p>0.05$; cohort 2: $t(14)=1.206$, $p>0.05$; cohort 3: $t(14)=0.358$, $p>0.05$).

Correlation of DAergic tone with behavior in NT+/+ and NT-/- mice

Overall % PPI values and pulse alone startle amplitudes for each individual subject were analyzed for correlation with physiological measurements for the HPLC experiment (Experiment 1), RT-PCR experiment (Experiment 2) and the autoradiography experiment (Experiment 3). For Experiment 2, $-dC_T$ values for gene expression targets were correlated with pulse alone amplitudes and overall %PPI values (summarized in Table 3-3). D1 mRNA was negatively correlated with overall % PPI in the NAcc in NT+/+ mice (Fig. 3-3a), while this correlation was absent in NT-/- mice (Fig. 3-3b and Table 3-3). Conversely, D1 mRNA was positively correlated with overall % PPI in the FCTX in NT+/+ mice (Fig. 3-3c), but was again not correlated with PPI in NT-/- mice (Fig. 3-3d and Table 3-3). There were no significant correlations between any of the gene expression targets and pulse alone startle amplitude (data not shown).

For Experiment 1, there were no significant correlations between % overall PPI or pulse alone startle amplitude and DA or DOPAC concentrations (data not shown). For Experiment 3, there were no significant correlations between % overall PPI or pulse alone startle amplitude and D1, D2, or DAT binding densities (data not shown).

3.5 DISCUSSION

The mesolimbic and nigrostriatal DA systems are known to display a great deal of plasticity in response to pharmacological (Belin *et al*, 2007; Neve *et al*, 1991; Shilling *et al*, 1997), genetic (Fauchey *et al*, 2000a; Jones *et al*, 1998), and developmental manipulations (Gatzke-Kopp, 2011). The experiments described in this chapter examined whether a deficit in NT produced developmental alterations in the mesolimbic and nigrostriatal DA systems by investigating DA and DA metabolite concentrations and DA receptor and transporter gene expression and binding in terminal regions in NT-/- mice. Indeed, these results show a lack of NT during development is associated with increases in striatal DA receptor and DAT gene expression and D2 binding. This leads

to the conclusion that NT is necessary for normal development of the mesolimbic and nigrostriatal DA systems in male mice.

Changes in gene expression and D2 binding in NT-/- mice

D1 receptor, D2 receptor, and DAT mRNA were significantly increased in the CP of NT-/- compared to NT+/+ mice. No significant changes in gene expression of these targets were noted in the FCTX or NAcc regions in NT-/- mice. DA denervation by MPTP treatment produces compensatory *increases* in striatal D1 receptor and D2 receptor mRNA (Smith *et al*, 1997), while constitutive hyperdopaminergia in a mutant mouse model (DAT knockout) produces compensatory *decreases* in D1 and D2 receptor expression (Fauchey *et al*, 2000b). As NT-/- mice have increased striatal D1 and D2 receptor expression, it might be hypothesized that mice lacking NT may show hypodopaminergic tone in the striatum. However, our studies showed no changes in DA concentrations, DOPAC concentrations, or DOPAC/DA ratio in any of the terminal regions in NT-/- mice compared to NT+/+ mice, indicating NT-/- mice do not show decreases in mesolimbic or nigrostriatal tonic DA or DA metabolism. Nonetheless, our study measured regional tissue concentrations to quantify tonic DA and DOPAC. It is possible that while tissue concentrations of DA and DOPAC are unchanged in NT-/- mice, synaptic concentrations of these monoamines or DA release might be altered. Follow-up experiments might utilize *in vivo* microdialysis or cyclic voltammetry to investigate these possibilities in NT-/- mice.

In light of our results from the gene expression study, D1 receptor, D2 receptor, and DAT binding in the dorsal and ventral striatum were investigated. D2-like, but not D1-like or DAT, binding was significantly elevated in the dorsal CP and NAcc shell in the absence of NT. Increases in D2-like binding most likely reflect increases in D2 receptor binding and not D3 or D4 receptor binding, as the autoradiography experiment was conducted in the presence of unlabeled 7-OH-DPAT to preclude D3 binding, and

raclopride (the radioligand used) has a low affinity for D4 receptors (Lahti *et al*, 1993). These results partially coincide with results from the gene expression study, as both D2 mRNA and D2 protein binding were elevated to a similar extent in the dorsal CP of NT-/- mice. However, in the binding experiment, NT-/- mice showed increased D2-like binding in the NAcc shell, while in the gene expression study, D2 mRNA in the NAcc was unaltered. This discrepancy might be due to differences in the methods used; in the gene expression study, D2 mRNA was measured by qRT-PCR from the dissected NAcc region while D2 binding was measured by autoradiography. Increased D2 binding in NT-/- mice may reflect increased D2 trafficking to the cell surface without increased D2 receptor expression and protein synthesis. In addition, NT-/- mice showed increased striatal D1 and DAT expression but did not show increases in striatal D1 and DAT binding. Increases in gene expression need not correlate with increases in protein, and it is possible that NT-/- have increased D1 and DAT mRNA but not increased D1 and DAT protein. It is also possible that NT-/- may have increased intracellular D1 and DAT protein levels but that these proteins are not trafficked to the cell surface and are thus not detected by autoradiography. Nevertheless, the results from the autoradiography experiment show that in the absence of NT, striatal D2 receptor levels are increased at cell surfaces.

Several studies have shown NT functionally opposes the effects of the D2 receptor via allosteric receptor/receptor interactions between the NTS1 receptor and D2 receptor. Activation of NTS1 by NT is known to desensitize and internalize D2 receptors (Thibault *et al*, 2011). In the absence of NT, it is thus not surprising that D2 binding is increased in the striatum. Striatal D2 receptors exist both pre-synaptically (D2S isoform) as autoreceptors as well as post-synaptically (D2L isoform). NT is able to desensitize both isoforms of the receptor *in vitro* and antagonize the function of both pre-synaptic and post-synaptic D2 receptors (Fuxe *et al*, 1992a; Thibault *et al*, 2011). From our

study, it is not possible to specifically determine whether D2 receptors were increased pre-synaptically or post-synaptically in NT-/- mice. Because the post-synaptic D2L receptor isoform comprises the vast majority of D2 receptor mRNA and protein in the striatum (Mack *et al*, 1991; Neve *et al*, 1991), we might theorize that an overall increase in D2 receptor binding density in the striatum may be due primarily to an increase in post-synaptic D2L receptors. However, future studies are needed to determine this. Follow-up experiments might utilize immunohistochemical methods to explore the cellular location of the increased D2 receptors in NT-/- mice.

Notably, from the DA receptor autoradiography study, alterations in striatal D2-like binding in NT-/- mice were apparent within discrete anatomical subdivisions of striatal regions. In NT-/- mice, D2 receptor binding densities were significantly increased in the NAcc shell, but not the NAcc core. In rodents, the NAcc core is more anatomically and functionally associated with the nigrostriatal DA system, whereas the NAcc shell is more associated with the mesolimbic DA system and extended amygdala (Heimer *et al*, 1997). In addition, the NAcc shell and core are distinct in regards to NT anatomy in rats; particularly, the NAcc shell receives mixed DA/NT projections from the VTA, while the NAcc core does not receive these mixed DA/NT afferents (Binder *et al*, 2001b). These distinctions between the NAcc core and shell and the results from our autoradiography study suggest that NT may play a more essential role in the development and function of the NAcc shell compared to the core.

The functional consequence of central NT injection is an antagonism of D2 function and a shift in post-synaptic DA transmission to D1 receptor-mediated effects (Jomphe *et al*, 2006). In NT-/- mice, striatal D2 receptors and the D2/D1 receptor ratio are increased. Thus, it might be predicted that a functional consequence of NT system disruption by NT gene knockout would be an increase in striatal D2 function and a decrease in D1-mediated neurotransmission. This theory is supported by behavioral

studies utilizing NT-/- mice. The acute hyperlocomotor effects of amphetamine and sensitization to amphetamine are attenuated in NT-/- mice (results in Chapter 2). Striatal post-synaptic D1 receptors facilitate amphetamine-induced hyperlocomotion and amphetamine sensitization (O'Neill *et al*, 1999), whereas striatopallidal D2 receptor-expressing neurons have an inhibitory effect on the reinforcing and locomotor effects of amphetamine (Durieux *et al*, 2009). Thus an increase in striatal D2 receptors in NT-/- mice may attenuate the acute hyperlocomotor effects of amphetamine and amphetamine sensitization. Finally, the effects of quinpirole (a D2-like agonist) are altered in NT-/- mice. In wildtype mice, intra-accumbal injection of quinpirole in mice enhances PPI (Mohr *et al*, 2007), an effect that contrasts with systemic administration of quinpirole, which has no effect on PPI in mice (Ralph-Williams *et al*, 2003; Ralph-Williams *et al*, 2002b). As reported in Chapter 2, systemic D2 receptor agonism in NT-/- mice had effects similar to intra-accumbal D2 receptor agonism in wildtype mice (increased PPI). This might be explained by the observation that NT-/- mice show increased accumbal D2 receptor expression that may bias the behavioral effects of systemic D2 receptor agonism. Taken together, these results suggest that NT-/- mice not only show increases in striatal D2 receptors but that this change has functional consequences for behavior. These conclusions are further discussed in the General Discussion section (Chapter 5).

Possible changes in relationship between cortical and subcortical tone

Interestingly, transient overexpression of striatal D2 receptors in mice results in deficits in prefrontal cortical DAergic neurotransmission (Kellendonk *et al*, 2006; Li *et al*, 2011). Cortical and striatal regions are connected anatomically directly through corticostriatal projections and indirectly through striatopallidothalamic projections (Alexander *et al*, 1986). In addition, both the ventral striatum and prefrontal cortex receive DAergic afferents from the VTA (Alexander *et al*, 1986), and experimental DAergic deafferentation of the prefrontal cortex in rodent models has been shown to

produce hyperactivity of DAergic function in the striatum, especially the ventral striatum (Deutch, 1993).

For this reason, DA and DOPAC concentrations were compared between NAcc and FCTX regions by ratio analyses in NT+/+ and NT-/- mice. While distinct regional concentrations of DA and DOPAC did not differ between NT+/+ and NT-/- mice, there was a significant increase in DOPAC/DA ratio in the NT-/- mice compared to the NT+/+ mice when these brain regions were compared ($p<0.05$) (Table 3-1). The DOPAC/DA ratio is thought to reflect DA utilization, so this result suggests there may be an increase in DA utilization in the NAcc relative to the FCTX in NT-/- mice compared to NT+/+ mice. This result may indicate a subtle imbalance in cortical and subcortical DAergic tone in NT-/- mice. Future studies might assess if there are changes in extracellular DA concentrations or DA release in the NAcc or FCTX of NT-/- mice compared to NT+/+ mice.

Correlation of DA receptor expression to sensorimotor gating

The results showed a strong negative correlation between individual differences in D1 mRNA and PPI in the NAcc and a positive correlation between D1 mRNA and PPI in the FCTX (Fig. 3-3). However, this correlation was absent in NT-/-. These results suggest frontal cortex D1 expression may positively modulate individual differences in sensorimotor gating, while accumbal D1 expression may negatively modulate individual differences in sensorimotor gating. In addition, these results suggest NT mediates the correlation between D1 expression and PPI, as there is a decoupling of D1 expression and sensorimotor gating in the absence of NT (in NT-/- mice). This result coincides with previous studies showing the D1 receptor plays an important role in regulating PPI in mice (Ralph-Williams *et al*, 2002a). However, while regional D1 mRNA was correlated with individuals differences in PPI in NT+/+ mice (Experiment 2), D1 binding was not correlated with PPI (Experiment 3). These results suggest D1 receptor binding does not

reflect D1 receptor gene expression, as was discussed above, either because the D1 message was not translated into protein or because the protein was not trafficked to the cell surface where it could be detected by autoradiography. Thus, the correlation between D1 mRNA and PPI might not reflect an actual relationship between the functional D1 receptor protein and behavior, but may instead reflect a subtle relationship between D1 receptor gene expression and sensorimotor gating behavior. Post-synaptic D1 receptor gene expression is regulated by synaptic concentrations of DA (Fauchey *et al*, 2000a; Gerfen *et al*, 1990), so the correlation between D1 expression and PPI might actually reflect a correlation between synaptic DAergic tone and PPI. Consistent with this theory, individual differences in PPI are negatively correlated with increased synaptic DAergic activity in the NAcc in rats (Yamada *et al*, 1998), and increasing DAergic activity in the NAcc via accumbal injection of DA disrupts PPI in rats (Swerdlow *et al*, 1992). In contrast, decreasing DAergic activity in the medial prefrontal cortex of rats decreases PPI (Ellenbroek *et al*, 1996). Thus, this result may indicate that DAergic activity correlates with PPI in the NAcc and frontal cortex in mice, but these correlations are absent in NT-/- mice, indicating a decoupling of DA systems and DA-sensitive behavior in the absence of NT. Future studies might test this by measuring synaptic concentrations of DA in NT+/+ and NT-/- mice and correlating this data with individual PPI measurements.

Relevance to schizophrenia

Some imaging studies show increased binding of D2-like receptors in the striata of people with schizophrenia (Seeman *et al*, 2000; Wong *et al*, 1997) and in their non-schizophrenic monozygotic twins, suggesting increased D2 receptor binding might be a biomarker for genetic susceptibility to schizophrenia (Hirvonen *et al*, 2005). However, other studies show no difference in D2-like binding between people with schizophrenia and healthy controls (Farde *et al*, 1990; Nordstrom *et al*, 1995). Furthermore, these

studies are sometimes confounded by patient use of antipsychotic drugs (D2 antagonists), which also upregulate D2-like binding (Burt *et al*, 1977). Nevertheless, many animal models of schizophrenia – including those that are altered genetically, pharmacologically, and developmentally – also show an increase in striatal D2 receptors (for review see Seeman *et al*, (2006)). The results from the present study show for the first time that NT deficiency during development is sufficient to increase D2 binding in the striata in mice, a schizophrenia-like phenotype. As previously mentioned, decreased levels of NT are found in the CSF of some schizophrenic patients (Breslin *et al*, 1994; Lindström *et al*, 1988; Sharma *et al*, 1997). These results suggest a decrease in NT in some patients with schizophrenia may be a causal factor in the observed increases in striatal D2 expression and in the development of psychosis.

In addition, DA dysregulation in striatal has been linked to DA dysregulation in cortical regions in the etiology of schizophrenia. Particularly, it is theorized that schizophrenia may be characterized by hyperdopaminergia in the mesolimbic system with concurrent hypodopaminergia in the mesocortical system (Davis *et al*, 1991; Deutch, 1993; Weinberger, 1987). The result showing DA utilization in the NAcc/FCTX in NT-/- compared to NT+/+ (Table 3-1) may be a subtle indicator of imbalance in cortical and subcortical DAergic tone. This observation further suggests NT system disruption might be involved in the disruption of DA circuits observed in schizophrenia. The value of the NT-/- mouse as a schizophrenia model is further discussed in the General Discussion section in Chapter 5.

FIGURES

Concentration (ng/mg total protein)			DA	DOPAC	DOPAC/DA
	n=6	**+/+**	135.5 ± 41.57	16.95 ± 4.02	0.157 ± .043
CP	***n=8***	**-/-**	160.3 ± 62.34	16.60 ± 4.72	0.123 ± 0.027
		***t* value**	0.30	0.05	0.50
		***p* value**	0.77	0.96	0.69
	n=8	**+/+**	123.4 ± 30.49	32.22 ± 9.37	0.271 ± 0.058
NAcc	***n=12***	**-/-**	85.54 ± 14.56	31.56 ± 5.95	0.402 ± 0.064
		***t* value**	1.27	0.06	1.37
		***p* value**	0.23	0.95	0.19
	n=7	**+/+**	1.67 ± 0.77	2.15 ± 0.93	1.61 ± 0.370
FCTX	***n=10***	**-/-**	1.68 ± 0.58	2.44 ± 0.84	1.21 ± 0.324
		***t* value**	0.01	0.22	0.81
		***p* value**	0.99	0.83	0.43
	n=6	**+/+**	345.5 ± 153.0	45.40 ± 17.27	0.171 ± 0.038
NAcc/FCTX	***n=9***	**-/-**	234.6 ± 118.0	240.9 ± 148.1	**0.665 ± 0.183***
Ratio		***t* value**	0.58	1.31	2.65
		***p* value**	0.57	0.23	<0.05

Table 3-1. Regional DA and DOPAC concentrations in NT+/+ and NT-/- mice.

Gene Expression (Fold Change)			D1 mRNA	D2 mRNA	DAT mRNA
	n=7	**+/+**	1.000 ± 0.053	1.000 ± 0.093	1.000 ± 0.127
Caudate	***n=8***	**-/-**	**1.605 ± 0.102*** **	**1.291 ± 0.080***	**1.602 ± 0.193***
Putamen		***t* value**	5.04	2.39	2.52
		***p* value**	<0.001	<0.05	<0.05
	n=8	**+/+**	1.000 ± 0.178	1.000 ± 0.088	1.000 ± 0.208
Nucleus	***n=8***	**-/-**	0.834 ± 0.091	0.868 ± 0.087	1.245 ± 0.194
Accumbens		***t* value**	0.83	1.07	0.86
		***p* value**	0.42	0.30	0.40
	n=8	**+/+**	1.000 ± 0.110	1.000 ± 0.162	1.000 ± 0.167
Frontal	***n=8***	**-/-**	0.801 ± 0.157	1.292 ± 0.225	3.518 ± 1.175
Cortex		***t* value**	1.04	1.05	1.98
		***p* value**	0.32	0.31	0.07

Table 3-2. Regional gene expression in NT+/+ and NT-/- mice.

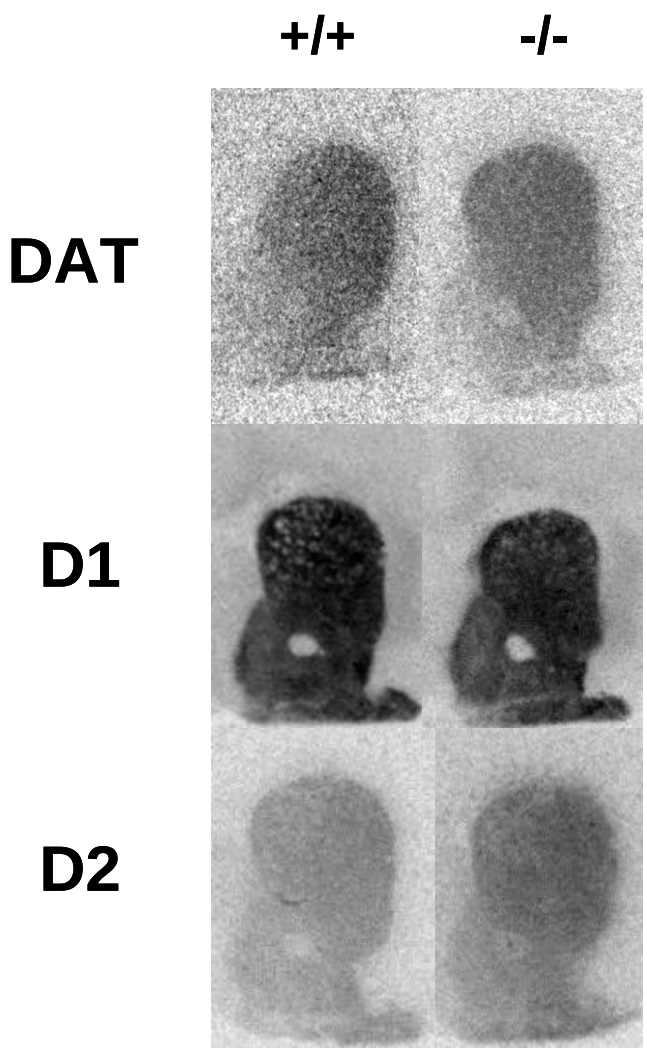

Fig. 3-1. Representative autoradiograms of coronal brain sections (Bregma AP +1.18) of NT+/+ and NT-/- mice. DAT binding, D1-like binding, and D2-like binding in NT+/+ and NT-/- mice.

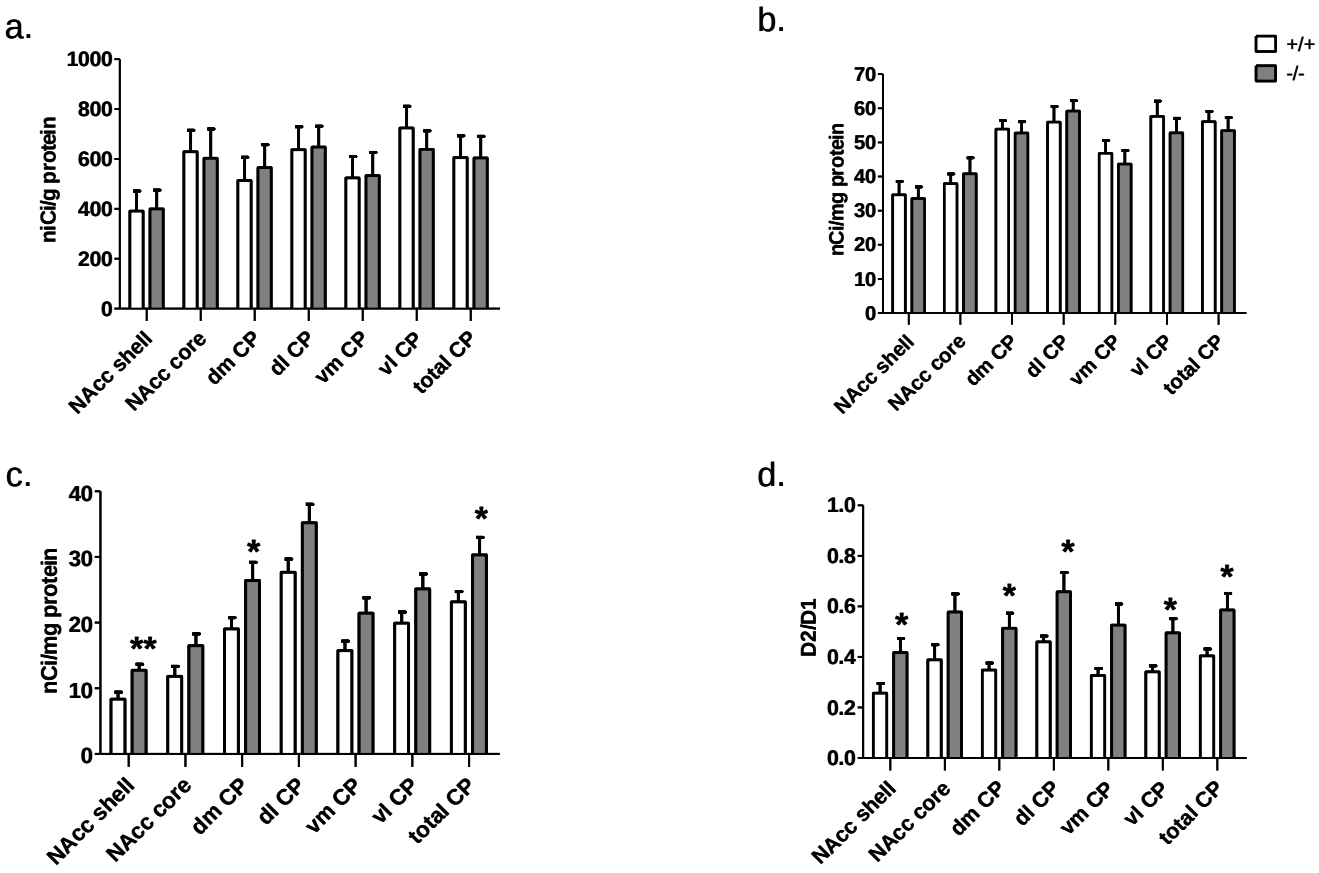

Fig. 3-2. DAT, D1-like, and D2-like binding in the NAcc. (a) DAT binding, (b) D1-like binding, (c) D2-like binding, and (d) D2/D1 ratio in NAcc (Bregma 1.18-1.1 mm) and CP (Bregma 1.18-0.98 mm) in NT+/+ and NT-/- mice. *p<0.05, **p<0.01, NT-/- compared to NT+/+ within brain region.

		NAcc			CP			FCTX		
Target	**Genotype**		**Pearson *r***	***p* value**		**Pearson *r***	***p* value**		**Pearson *r***	***p* value**
DAT mRNA	**All**		0.241	0.37		-0.069	0.81		0.066	0.81
	+/+	***n=8***	0.498	0.21	***n=7***	-0.522	0.23	***n=8***	-0.166	0.72
	-/-	***n=8***	-0.185	0.66	***n=8***	-0.106	0.80	***n=8***	-0.305	0.46
D1 mRNA	**All**		-0.337	0.20		0.170	0.54		0.119	0.66
	+/+		**-0.848**	**<0.001****		0.265	0.57		**0.736**	**<0.05***
	-/-		0.055	0.90		-0.386	0.34		-0.043	0.92
D2 mRNA	**All**		0.001	0.99		-0.036	0.90		0.136	0.62
	+/+		0.518	0.19		-0.178	0.70		0.520	0.19
	-/-		-0.255	0.54		-0.300	0.47		-0.325	0.42

Table 3-3. Correlation of mRNA and overall % PPI values in NT+/+ and NT-/- mice.

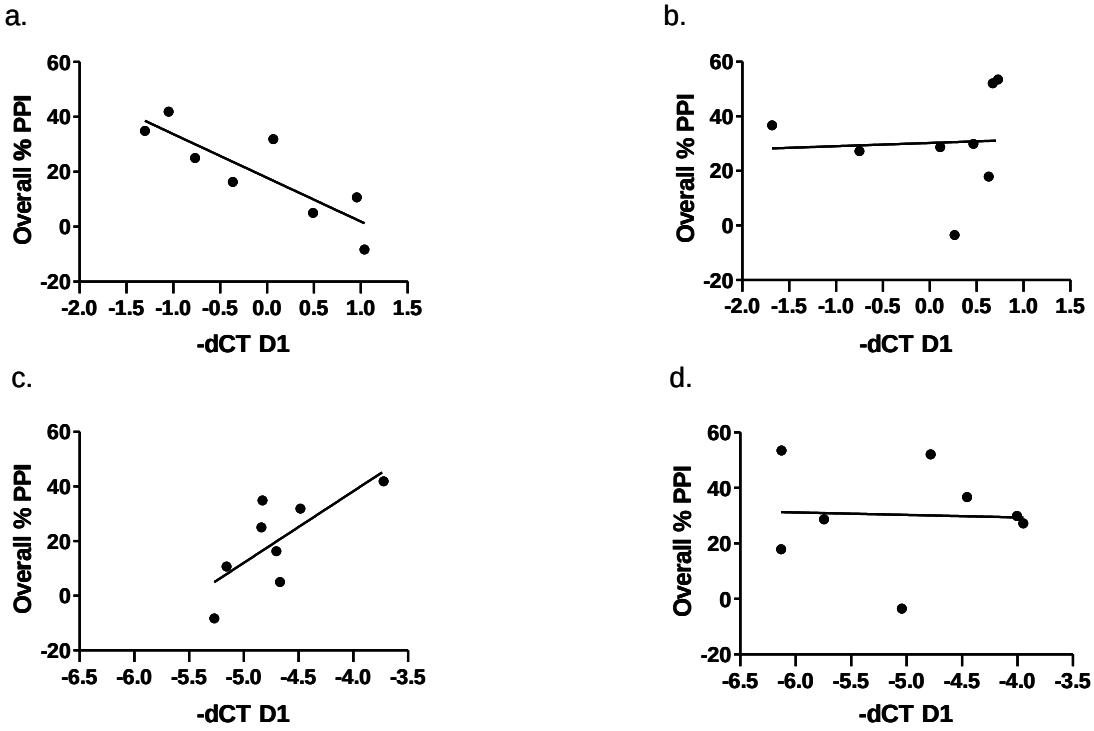

Fig. 3-3. Correlation of D1 mRNA and overall % PPI values in NT+/+ and NT-/- mice. D1 mRNA was negatively correlated with overall % PPI in the NAcc in NT+/+ mice (a), while this correlation was absent in NT-/- mice (b). D1 mRNA was positively correlated with overall % PPI in the FCTX in NT+/+ mice (c), but was again not correlated with PPI in NT-/- mice (d).

4. THE CONSEQUENCES OF NEUROTENSIN DEFICIENCY IN FEMALE MICE

4.1 ABSTRACT

Rodents show sex differences in basal DA system activity, behavioral sensitivity to DA agonists, and DA-mediated behaviors. As previous studies show, NT plays an important role in modulating the effects of DA agonists on locomotor behavior and sensorimotor gating (Chapter #2) and in regulating striatal DA receptor expression (Chapter #3). Male mice lacking NT (NT-/-) previously showed baseline deficits in sensorimotor gating (PPI). Unlike male NT-/- mice, female NT-/- mice did not show a deficit in baseline PPI. Studies show ovarian hormones are protective against PPI disruption in rats. Thus, it is possible that the effects of NT gene knockout might be sex-specific, and being female may be protective against the disruptive effects of NT gene knockout on the behavioral response to amphetamine and DA receptor binding. This hypothesis was tested in the following experiments. Male NT-/- mice previously showed diminished response to acute and repeated amphetamine administration (Chapter #2) and increased striatal D2-like binding (Chapter #3). In contrast, in female NT-/- mice, the effects of acute and repeated amphetamine administration on locomotor behavior were not different from those in female NT+/+ mice. In addition, female NT-/- mice, unlike male NT-/- mice, did not show significant alterations in D2-like striatal binding compared to controls. However, female NT-/- mice showed significantly decreased D1-like binding in the NAcc shell compared to female NT+/+ mice, a developmental modification not observed in male NT-/- mice. The decrease in D1-like binding in female NT-/- mice was not affected by estrous cycle phase. Thus, the consequences of NT gene knockout are sexually dimorphic. Being female may have a protective effect against some of the effects of NT knockout, but NT knockout also produces discrete physiological alterations in female mice.

4.2 INTRODUCTION

The prevalence of schizophrenia is about equal in men and women, but the age of onset and outcome of the disease vary between genders. Epidemiological studies show the age of onset of schizophrenia in men peaks sharply in young adulthood between the ages of 15-25, while the age of onset of schizophrenia in women peaks later and more gradually between the ages of 15-30. Studies also show a second, somewhat smaller peak in age of onset in women between the ages of 40-45 around premenopause, whereas men do not show a second peak in age of onset (Castle *et al*, 1993; Hafner, 2003). In addition, women with schizophrenia show a better response to treatment with antipsychotic drugs than men (Castle *et al*, 1995; Szymanski *et al*, 1995). Some studies show sex differences in symptomatology in schizophrenia; particularly men tend to show more severe cognitive and negative symptoms, while women tend to show more atypical and affective symptoms (Castle *et al*, 1995; Goldstein *et al*, 1998). However, not all studies have replicated these findings (Hafner, 2003). The neurobiological basis for these sex differences is unknown. One theory suggests ovarian hormones, particularly estrogen, may be protective against schizophrenia, and may explain the rise in psychotic onsets around menopause in women and the more favorable treatment outcome in women compared to men (Hafner, 2003; Huber *et al*, 2004; Rao and Kolsch, 2003). Another theory suggests these sex differences actually reflect different subtypes of schizophrenia that affect males and females differentially, possibly due to differences in neurodevelopment or genetic factors (Castle *et al*, 1995). These unknowns highlight the necessity to further explore the biology of sex differences relevant to schizophrenia.

As previously described in the Introduction, clinical studies suggest deficits in NT neurotransmission may be involved in the etiology of schizophrenia in at least a subset of patients (Breslin *et al*, 1994; Lindström *et al*, 1988; Nemeroff *et al*, 1989a; Sharma *et*

al, 1997; Widerlöv *et al*, 1982). In addition, clinical and experimental evidence suggests dysfunction in the mesolimbic DA system, which is modulated by NT, is a key facet in the neuropathology of schizophrenia (Grace, 1991; Seeman, 1987; Seeman *et al*, 2000). Studies in animal models note important sex differences in both the DA and NT systems. Female rodents show differences from males in basal striatal DA concentrations and amphetamine-stimulated DA release (Becker and Cha, 1989; Xiao and Becker, 1994), striatal DA receptor densities (Andersen and Teicher, 2000), and striatal DAT function and DAT densities (Bhatt and Dluzen, 2005; Morissette and Di Paolo, 1993). In addition, female rats show differences in behavioral responses to amphetamine and DA receptor agonists compared to males (Camp and Robinson, 1988; Robinson *et al*, 1982). Studies suggest some of these sex differences are mediated by ovarian hormones as they are abolished by ovariectomy and by natural changes in ovarian hormones during estrous cycling (Becker *et al*, 1989; Camp *et al*, 1986). Rats also show sexual dimorphism in NT gene expression in the hypothalamus and caudate and in NT peptide levels in the VTA (Alexander *et al*, 1991; Kinkead *et al*, 2000).

Male mice lacking NT (NT-/-) have altered DA receptor gene expression and density (Chapter 3) and behavioral response to DA receptor activation (Chapter 2). Male NT-/- mice were previously shown to have deficits in PPI, a behavior regulated by mesolimbic DA activity (Kinkead *et al*, 2005). Interestingly, female NT-/- mice do not show disrupted PPI suggesting the consequences of NT gene knockout are sexually dimorphic (Kinkead *et al*, 2005). The experiments described in this chapter investigated the effects of NT deficiency on locomotor response to amphetamine (Experiment #1) and striatal DA receptor binding density (Experiment #2) in *female mice*. Studies show ovarian hormones are protective against pharmacological PPI disruption in rats (Bubeníková *et al*, 2005; Gogos and Van den Buuse, 2004). Thus, it was hypothesized that the effects of NT gene knockout might be sex-specific, and being female may be

protective against the disruptive effects of NT gene knockout on the behavioral response to amphetamine and DA receptor binding densities.

4.3 METHODS

Animals

NT knockout mice were generated as previously described in Dobner *et al.* (2001) and in Chapter #2. Only female mice (60 days of age and older) from the lab's NT knockout breeding colony were used. Animals were housed in an environmentally-controlled animal facility with a reversed 12 hour light-dark cycle (lights off at 10:00 AM; lights on at 10:00 PM). All behavioral testing and euthanasia procedures were completed in the dark phase between 10:00 AM and 5:00 PM. All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee (IACUC) in compliance with the National Institutes of Health.

Genotyping

Animals were genotyped using the same procedures described in Chapter #2.

Locomotor Testing

For Experiment #1, locomotor activity measurements were evaluated in an open field as described in Chapter #2. Activity was video-recorded for either 30 min or 60 min, and videotapes were post-processed to quantify time-dependent spontaneous behavior.

Estrous Cycle

For experiment #2, estrous cycle was determined by vaginal lavage daily at 10:00 AM. Based on the number and type of cell present in the vaginal swab, females were categorized into five groups: diestrus 1, diestrus 2, proestrus, estrus, and metestrus. Only females showing typical cycles were used in this study.

Experiment #1

Effects of amphetamine on locomotor behavior

Two different cohorts underwent locomotor testing. Both cohorts received the same doses of amphetamine (2 mg/kg). Cohort #1: Female NT+/+ (*n*=8) and NT-/- (*n*=7) mice were tested for the acute locomotor effects of amphetamine (2 mg/kg s.c.) Animals first underwent baseline testing and one week later the effects of acute amphetamine on locomotor activity were examined. Testing sessions were 30 min. Cohort #2: Female NT+/+ (*n*=6) and NT-/- (*n*=7) mice were tested for the acute and repeated effects of amphetamine (2 mg/kg s.c.) All animals underwent all tests and treatments in a within-subjects design. Animals were first tested for baseline locomotor activity. A week after baseline testing, the effects of amphetamine on locomotor activity were examined on two consecutive days (amphetamine treatment #1 and amphetamine treatment #2). Seven days after the 2nd treatment, animals received a third injection of amphetamine (amphetamine treatment #3) and locomotor activity was examined. All testing sessions were 60 min. The experimental protocol is summarized in Fig. 2-1.

Experiment #2

Receptor binding autoradiography

Estrous cycles were determined in female NT+/+ and NT-/- mice (*n*=11/genotype). Animals determined to be in diestrus or proestrus, days in which ovarian hormones are low or high respectively (see Fig. 4-1), were euthanized by rapid decapitation (*n*=5-6/cycle/genotype). Brains were collected and quickly frozen on dry ice. Brains were sectioned on a cryostat at 25 µm thickness and mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA). Slides were stored at -70ºC until autoradiography, and alternate sections from the same brains were used for separate binding assays. D1 and D2 receptor binding assays were conducted as described in Chapter #3. Slides were exposed to BAS-5000 phosphoimaging plates (FujiFilm) for 48 hours (D1 binding) or 12

days (D2 binding) along with tritium standards (American Radiolabeled Chemicals, Inc., St. Louis, MO).

To determine regions of interest, slide-mounted sections were dyed using Neutral Red and were compared to a mouse atlas (Franklin *et al*, 1997). For all sections, two densitometry measurements were made in the NAcc bilaterally, one at bregma +1.18 mm and one at bregma +1.1 mm. Densitometries were sampled in the NAcc core in a rectangular area and in the NAcc shell using the free hand tracing tools in MultiGauge and AIS. The CP was measured bilaterally at bregma +1.1 mm and +0.98 mm. Densitometries were sampled in CP subregions in rectangular areas and in the total CP using the free hand tracing tools in MultiGauge and AIS. Background was subtracted from regions of interest from adjacent areas lacking specific binding on the same section.

Statistical Analysis

Data were tested for normality using the Shapiro-Wilk test. In all cases, the data passed the Shapiro-Wilk test, indicating the samples were from a Gaussian distribution. Thus, in all analyses, parametric statistical tests were utilized. For experiment #1 and #2, ANOVAs were utilized. Following ANOVAs, planned comparisons were tested using Tukey's HSD post-test.

For experiment #1, the two cohorts were analyzed separately. Since the experiments utilized a within-subjects design, two-way repeated measures ANOVAs (genotype x drug) was used to analyze the effect of acute amphetamine on distance moved (m) by comparing the baseline session (no drug) to the first amphetamine treatment session. For the subchronic amphetamine analyses (cohort #2), two-way repeated measures ANOVAs were used to compare the first and second amphetamine treatment sessions to the third amphetamine treatment session to measure locomotor sensitization to amphetamine. For experiment #2, two-way ANOVAs (genotype x

estrous cycle) were used to analyze the effects of genotype and estrous cycle stage on receptor binding densities. D1 and D2 densities were analyzed separately. Tukey's post-tests were used for planned pairwise comparisons.

4.4 RESULTS

Effects of acute and subchronic amphetamine on locomotor behavior in female NT+/+ and NT-/- mice

For cohort #1, receiving 2 mg/kg acute amphetamine (Fig. 4-2a), a two-way repeated measures ANOVA (genotype x drug) for the acute amphetamine analysis showed a significant effect of drug ($F(1,13)=14.545$, $p<0.01$), no significant effect of genotype ($p>0.05$), and no genotype x drug interaction ($p>0.05$). Tukey's tests comparing the genotypes showed baseline locomotor behavior (distance moved) and locomotor response to the first amphetamine treatment were not significantly different between NT+/+ and NT-/- mice ($p>0.05$). Pairwise comparisons within each genotype showed acute amphetamine significantly increased distance moved compared to baseline ($p<0.05$), indicating amphetamine increased locomotor activity in both genotypes. These results indicate there were no differences between female NT+/+ and NT-/- in baseline locomotor behavior or acute locomotor response to 2 mg/kg amphetamine.

For cohort #2, receiving 2 mg/kg acute and repeated amphetamine (Fig. 4-2b), a two-way repeated measures ANOVA (genotype x drug) for the acute amphetamine analysis showed a significant effect of drug ($F(1,11)=8.163$, $p<0.05$), no significant effect of genotype ($p>0.05$), and no genotype x drug interaction ($p>0.05$). Tukey's tests comparing the genotypes showed baseline locomotor behavior (distance moved) and locomotor response to the first amphetamine treatment were not significantly different between NT+/+ and NT-/- mice ($p>0.05$). Pairwise comparisons within each genotype

showed acute amphetamine increased distance moved compared to baseline, a trend which approached significance for both NT+/+ ($p=0.084$) and NT-/- mice ($p=0.055$). As with cohort #1, these results indicate there were no differences between female NT+/+ and NT-/- in baseline locomotor behavior or acute locomotor response to 2 mg/kg amphetamine.

For the amphetamine sensitization analyses for cohort #2 (Fig. 4-2b), a two-way repeated measures ANOVA (genotype x drug) showed a significant effect of drug ($F(1,11)=5.147$, $p<0.05$). The effect of genotype and the genotype x drug interaction were not significant ($p>0.05$). Tukey's tests comparing the genotypes showed locomotor response to amphetamine #2 and amphetamine #3 were not significantly different between NT+/+ and NT-/- mice ($p>0.05$). Tukey's tests within the NT+/+ mice and NT-/- mice comparing the first amphetamine injection with the third amphetamine injection did not reach significance ($p>0.05$). A two-way ANOVA (genotype x drug) comparing distance moved after the second amphetamine injection (amphetamine #2) and the third amphetamine injection (amphetamine #3) did not show a significant effect of drug ($p>0.05$), genotype ($p>0.05$), or a genotype x drug interaction ($p>0.05$). These results indicate the effects of repeated 2 mg/kg amphetamine were similar in female NT+/+ and NT-/- mice.

DA receptor binding in female NT+/+ and NT-/- mice

Differences in D1-like binding densities in the NAcc shell, NAcc core, and CP in female NT+/+ and NT-/- mice were examined (Fig. 4-3). To control the possible effect of ovarian hormones, samples were collected at diestrus 1 and proestrus, days in the estrous cycle in which ovarian hormones are low or high respectively (see Fig. 4-1). No differences in estrous cycle quality or regularity were noted in NT-/- mice compared to NT+/+ mice. For D1-like binding in the NAcc shell (Bregma 1.1mm but not Bregma 1.18mm), a two-way ANOVA (genotype x estrous cycle) showed a significant effect of

genotype ($F(1,18)=4.590$, $p<0.05$). There was no significant effect of estrous cycle and no interaction ($p>0.05$). However, Tukey's tests comparing D1-like binding in the animals within each estrous cycle stage showed no significant differences between NT+/+ and NT-/- mice. These results indicate NT-/- mice show a slight decrease in D1-like binding in the NAcc shell compared to NT+/+ mice which is independent of estrous cycle (Fig. 4-3a). In the NAcc core, there were no significant differences in D1-like binding between NT+/+ and NT-/- mice (Fig. 4-3b). In the CP, a two-way ANOVA (genotype x estrous cycle) showed a significant effect of estrous cycle ($F(1,18)=5.203$, $p<0.05$). There was no significant effect of genotype and no interaction ($p>0.05$). Tukey's tests comparing D1-like binding in the CP within each genotype showed no significant differences between diestrus 1 and proestrus. These results indicate a slight increase in D1-like binding in proestrus compared to diestrus, regardless of genotype (Fig. 4-3b).

Differences in D2-like binding densities were also examined (Fig. 4-4). In the NAcc shell (Bregma 1.1mm and 1.18mm), a two-way ANOVA (genotype x estrous cycle) showed statistical trends for the effect of genotype, but these trends did not reach significance ($p=0.09$) (Fig. 4-4a). There was no effect of estrous cycle on D2 binding in the NAcc shell ($p>0.05$). In the NAcc core (Fig. 4-4b) and CP (Fig. 4-4c), there were no significant differences in D2-like binding between NT+/+ and NT-/- mice, and there were no effects of estrous cycle. These results indicate there were no significant differences in striatal D2-like binding between female NT+/+ and NT-/- mice.

4.5 DISCUSSION

The experiments described in this chapter sought to investigate whether female mice, like male mice, show functional and/or physiological alterations in the striatal DA system as a consequence of NT deficiency during development. Male NT-/- mice

previously showed diminished acute hyperlocomotor response to amphetamine (2 mg/kg) and decreased sensitization to the locomotor response to amphetamine (1 mg/kg) compared to NT+/+ mice (Chapter 2). In contrast, female NT-/- mice did not show differences in hyperlocomotor response to amphetamine or amphetamine sensitization compared to NT+/+ mice at the 2 mg/kg dose (Fig. 4-2). Male NT-/- mice also showed increased D2-like binding densities in the NAcc shell and CP compared to male NT+/+ mice (Chapter 3), while female NT-/- mice, did not show significant differences in D2-like binding in any striatal region compared to female NT+/+ mice (Fig. 4-4). However, female NT-/- showed decreased D1-like binding density in the NAcc shell compared to female NT+/+ mice, a result not observed in male NT-/- mice (Fig. 4-3). These results indicate that the developmental consequences of NT gene knockout on striatal DA system physiology are sexually dimorphic.

Locomotor response to amphetamine in female NT+/+ and NT-/- mice

Unlike male NT-/- mice, female NT-/- mice did not show a diminished hyperlocomotor response to amphetamine or diminished amphetamine sensitization compared to NT+/+ mice (Fig. 4-2). Notably, in our studies utilizing female mice, the locomotor-stimulating effects of amphetamine were only tested at the 2 mg/kg dose; thus the results of these studies cannot be directly compared to the results of the male studies utilizing 1 mg/kg amphetamine. It is possible, that, if the 1 mg/kg dose were tested in female mice, the results might show a difference between genotypes. Nonetheless, a direct comparison between female and male mice may be made for the results of the studies utilizing 2 mg/kg amphetamine. While male NT-/- mice lacked a significant acute hyperlocomotor response to 2 mg/kg amphetamine, female NT-/- mice, similar to NT+/+ mice, showed a significant hyperlocomotor response to amphetamine at this dose. These studies indicate a sex difference in the behavioral effects of NT deficiency.

These studies are congruent with the literature on sex differences in behavioral response to amphetamine in rats. Female rats show increased behavioral and physiological responses to amphetamine compared to males (for review see Anker and Carroll (2010)). Particularly, female rats show greater and more rapid susceptibility to amphetamine sensitization than males (Camp *et al*, 1988). In line with these studies, NT gene knockout diminished the locomotor effects of amphetamine in males but not females. These studies suggest NT modulates the acute amphetamine response and amphetamine sensitization in males, but not females, and, thus, the functional role of NT in regards to locomotor response to amphetamine is sex-specific.

The biological basis for this observed sex difference in the effect of NT gene knockout on amphetamine response is unknown. Many studies suggest ovarian hormones enhance the effects of amphetamine. In rats, behavioral and physiological responses to amphetamine increase during estrus, a time when ovarian hormones are high (Becker *et al*, 1989). In addition, ovariectomy decreases responses to amphetamine (Camp *et al*, 1986), an effect that is rescued by administration of estrogen (Becker, 1990). Thus, it is possible that NT plays an important role in modulating the behavioral response to amphetamine in males, but the presence of high concentrations of ovarian hormones (in females) may compensate for the loss of NT in this function. This study did not control for the possible effects of ovarian hormones, and future studies might address this possibility by examining amphetamine response across the animals' estrous cycles. Follow-up experiments might also address the possibility that ovarian hormones may block the behavioral consequences of NT deficiency by utilizing ovariectomized NT+/+ and NT-/- females. Another possible explanation for these sex differences might be that NT knockout causes different developmental alterations in the DA systems of males and female mice, which results in different behavioral outcomes. This possibility was investigated in Experiment #2, and is discussed below.

DA receptor binding density in female NT+/+ and NT-/- mice

Male NT-/- mice show altered behavioral responses to amphetamine (Chapter 2), disrupted PPI (Kinkead *et al*, 2005), and altered striatal DA receptor binding densities (Chapter 3). Locomotor response to amphetamine (Pennartz *et al*, 1994; Swerdlow and Koob, 1985) and PPI (Swerdlow *et al*, 2001; Yamada *et al*, 1998) are regulated by striatal DA activity. In light of the lack of behavioral alterations in female NT-/- mice (normal hyperlocomotor response to amphetamine and normal PPI), it was hypothesized that female NT-/- mice might also lack the developmental alterations in striatal DA receptor density observed in male NT-/- mice. Indeed, female NT-/- mice did not show increased striatal D2 binding densities as male NT-/- mice did (Fig. 4-4). This observation supports the theory that the behavioral changes observed in male NT-/- mice (diminished response to amphetamine and disrupted PPI) are due to developmental increases in striatal D2 receptors due to NT deficiency. This theory is further explored in the General Discussion in Chapter 5.

Unexpectedly female NT-/- mice showed small but significant decreases in D1-like densities in the NAcc shell, a result not observed in male NT-/- mice (Fig. 4-3). These decreases were independent of estrous cycle stage suggesting this developmental alteration was not mediated by the effect of ovarian hormones. It is unknown whether the decreased accumbal D1-like densities in female NT-/- mice have functional significance. It might be expected that decreased accumbal D1 receptor availability would result in diminished locomotor response to amphetamine, but this effect was not observed in female NT-/- mice. Future studies might utilize selective D1 receptor agonists like SKF-82958 to examine possible changes in the behavioral effects of D1 receptor activation in female NT-/- mice.

Notably, dorsal striatal D1-like binding densities were found to be increased in proestrus compared to diestrus in female mice independent of genotype (Fig 4-3c).

These results contrast with a study in rats that showed striatal D1 binding densities peak at diestrus 2 compared to other estrous cycle stages (Levesque *et al*, 1989). These results may reflect a species difference in fluctuations in D1 densities across estrous cycles. However, our study showed striatal D2 densities as measured by antagonist binding in female mice did not vary across estrous cycle, an observation that concurs with a receptor binding study in female rats (Di Paolo 1988).

Clinical relevance

In a previous study, female NT-/- mice did not show disruptions in baseline PPI as did male NT-/- mice (Kinkead *et al*, 2005). In the studies presented in this chapter, female NT-/- mice did not show increased striatal D2-like binding densities. Diminished sensorimotor gating (Kumari *et al*, 2000) and increased striatal binding of D2-like receptors are both physiological disruptions observed in patients with schizophrenia (Hess *et al*, 1987; Hirvonen *et al*, 2005; Seeman *et al*, 2000; Wong *et al*, 1997). Interestingly, some studies report only male schizophrenic patients (not female patients) show diminished PPI in comparison to sex-matched controls (Kumari *et al*, 2004), and as mentioned in the Introduction to this chapter, some studies suggest men with schizophrenia show more severe symptoms than women with the disease (Castle *et al*, 1995; Goldstein *et al*, 1998). Thus, the results obtained in our animal model coincide with observations in clinical studies of schizophrenia, in that, female mice are 'protected' from some of the consequences of NT deficiency.

On the other hand, female NT-/- mice show a significant decrease in D1-like binding densities in the NAcc shell compared to female NT+/+ mice, a result that was not observed in male NT-/- mice. Most clinical studies have not found changes in D1 receptor density in schizophrenia (Joyce *et al*, 1988; Pimoule *et al*, 1985; Seeman *et al*, 1987b), but one study reported decreases in striatal D1 density in post-mortem brains from schizophrenic patients (Hess *et al*, 1987). Thus, our results showing a decrease in

D1-like binding in female NT-/- mice in our study might be relevant to some cases of schizophrenia. Several lines of research have suggested cognitive deficits in schizophrenia may involve hypofunctional D1 receptors (Davis *et al*, 1991; Weinberger, 1987). Future studies in our lab might assess whether decreases in striatal D1 receptor density in female NT-/- mice have functional consequences.

Many studies suggest ovarian hormones, particularly estrogen, are protective against more severe symptoms in schizophrenia (Hafner, 2003; Huber *et al*, 2004; Rao *et al*, 2003). In our study, the effect of estrous cycle did not mediate the effects of NT knockout on female mice. Particularly, there were no differences in striatal D2 receptor densities in either genotype across the estrous cycle, and the decreases observed in D1 receptor densities in NT-/- mice were independent of the effect of estrous cycle. These data suggest that ovarian hormones do not explain the observed sex differences in the effects of NT knockout on striatal DA receptor binding densities. Interpreted as an animal model of schizophrenia, these data do not support the 'ovarian hormone protection theory' for sex differences in schizophrenia, but, instead, support the theory that sex differences in schizophrenia are due to sex-specific subtypes of the disease that affect males and females differentially, possibly due to differences in neurodevelopment (Castle *et al*, 1995). Indeed, male and female rats show differences in striatal DA receptor quantities throughout development suggesting the maturation of striatal DA innervation is sexually dimorphic (Andersen *et al*, 2000). Thus, it is possible NT deficiency during development might differentially affect striatal DA receptor expression and availability in males and females. Nonetheless, it may be possible that the presence of sex hormones during development and/or throughout adulthood might influence the distinct sex-specific effects of NT deficiency observed in these studies. Follow up experiments are needed to address these questions. Future experiments might further scrutinize the role of sex hormones in the sex differences observed in NT-/- mice by

utilizing NT+/+ and NT-/- mice that are gonadectomized pre-puberty and during adulthood.

While clinical studies on sex differences in schizophrenia suggest women may fare better in terms of symptom severity and treatment outcome, clinical studies on drug abuse suggest women are more vulnerable to stimulant abuse and addiction than men. Particularly women develop drug addiction more quickly and show greater rates of relapse than men (Becker and Hu, 2008; Brady and Randall, 1999; Lynch *et al*, 2002). Our studies coincide with these clinical observations in that NT gene knockout diminished acute amphetamine response and amphetamine sensitization in male but not female mice. These results suggest NT might play an important role in acute response to amphetamine and amphetamine-induced behavioral plasticity in males but not females. Whether the role of NT in the rewarding effects of amphetamine and amphetamine addiction is sex-specific needs to be tested in follow up experiments. Future experiments might utilize different experimental paradigms predictive of amphetamine addiction such as amphetamine conditioned place preference or amphetamine self-administration paradigms.

FIGURES

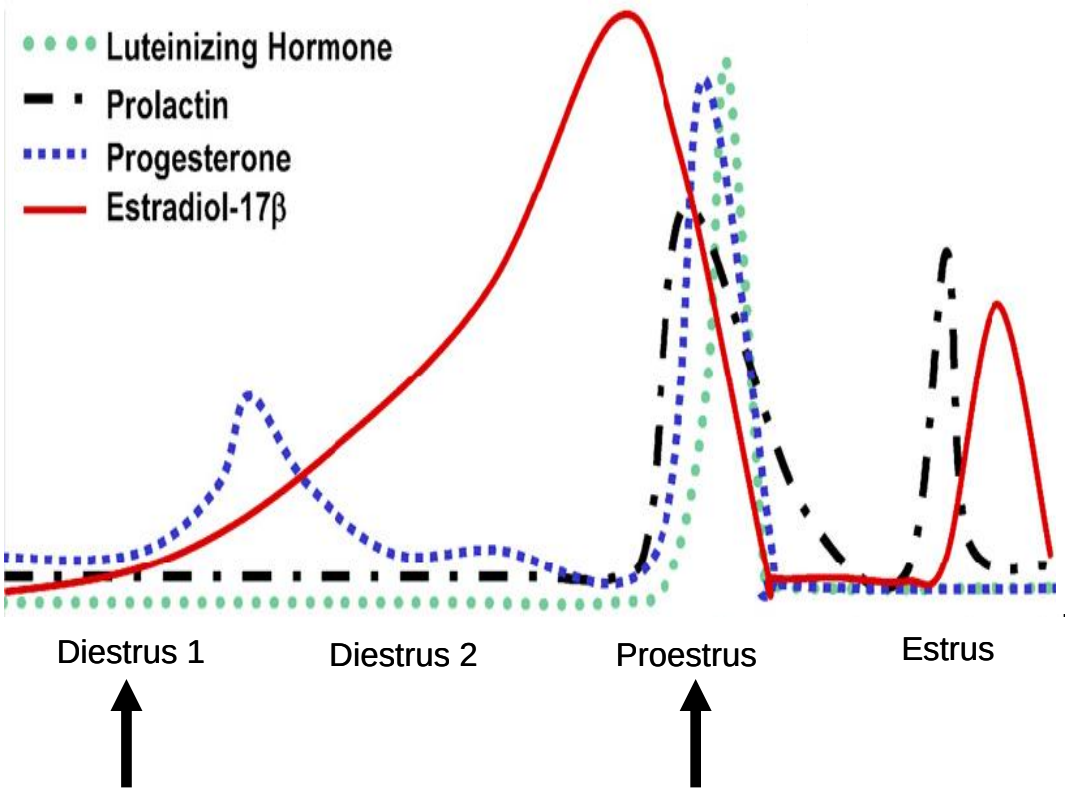

Fig. 4-1. Diagram of ovarian hormones during the rodent estrous cycle. Brain samples were taken at diestrus 1 and proestrus. Diagram modified from Kinkead *et al*. (2008b).

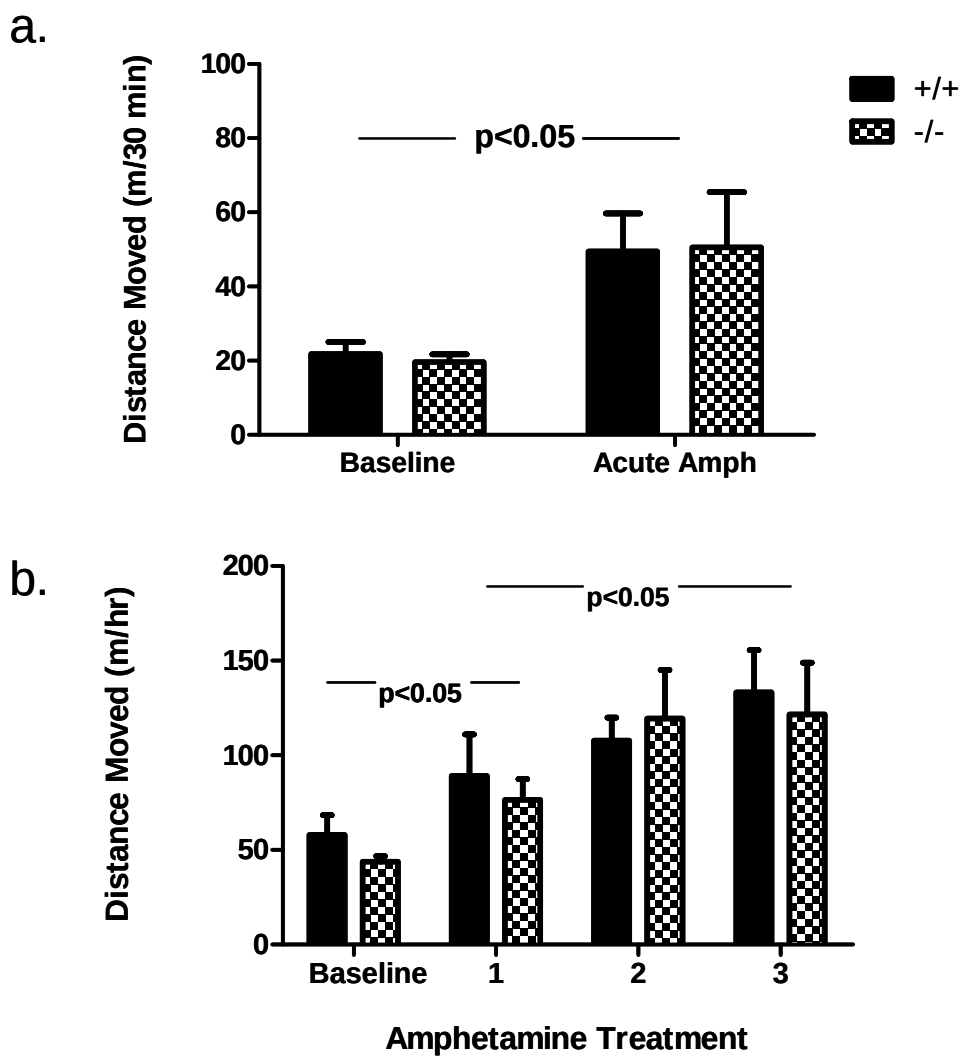

Fig. 4-2. The (a) acute and (b) subchronic effects of amphetamine (2 mg/kg) on distance moved in female NT+/+ and NT-/- mice. Data are expressed as mean distance moved (m) in 30 min (a) or in 1 hour (b) ± S.E.M. For acute locomotor response to amphetamine (a & b), two-way ANOVAs showed a significant effect of drug ($p<0.05$) but no effect of genotype, indicating both genotypes showed an acute hyperlocomotor response to amphetamine, and NT+/+ and NT-/- mice did not differ in acute locomotor response to amphetamine. Likewise, comparing Amphetamine 1 and 2 (b), there was a significant effect of drug ($p<0.05$), but no effect of genotype, indicating both genotypes

sensitized to the locomotor effects of amphetamine, and NT+/+ and NT-/- mice did not differ in locomotor response to repeated amphetamine.

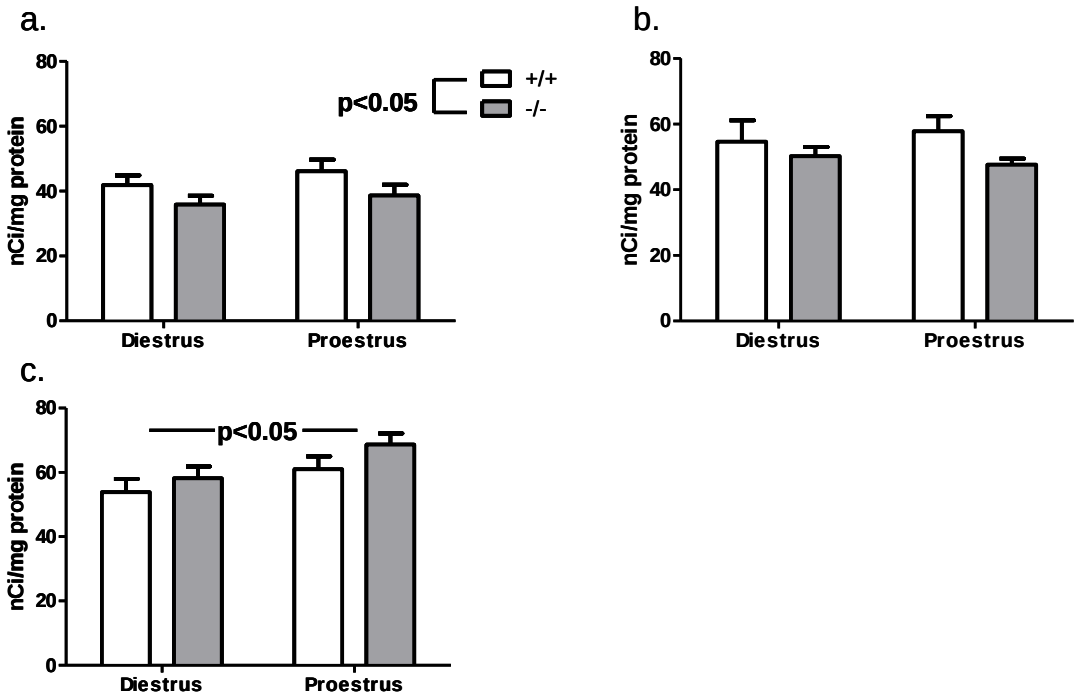

Fig. 4-3. D1-like binding in the (a) NAcc shell (b) NAcc core and (c) CP in female NT+/+ and NT-/- mice. Data are expressed as mean binding density (nCi/mg protein) ± S.E.M. (a) NT-/- mice showed less D1-like binding in the NAcc shell compared to NT+/+ mice. (b) There were no differences in D1-like binding between NT+/+ and NT-/- mice in the NAcc core. (c) There was increased D1-like binding in the CP during proestrus compared to diestrus regardless of genotype.

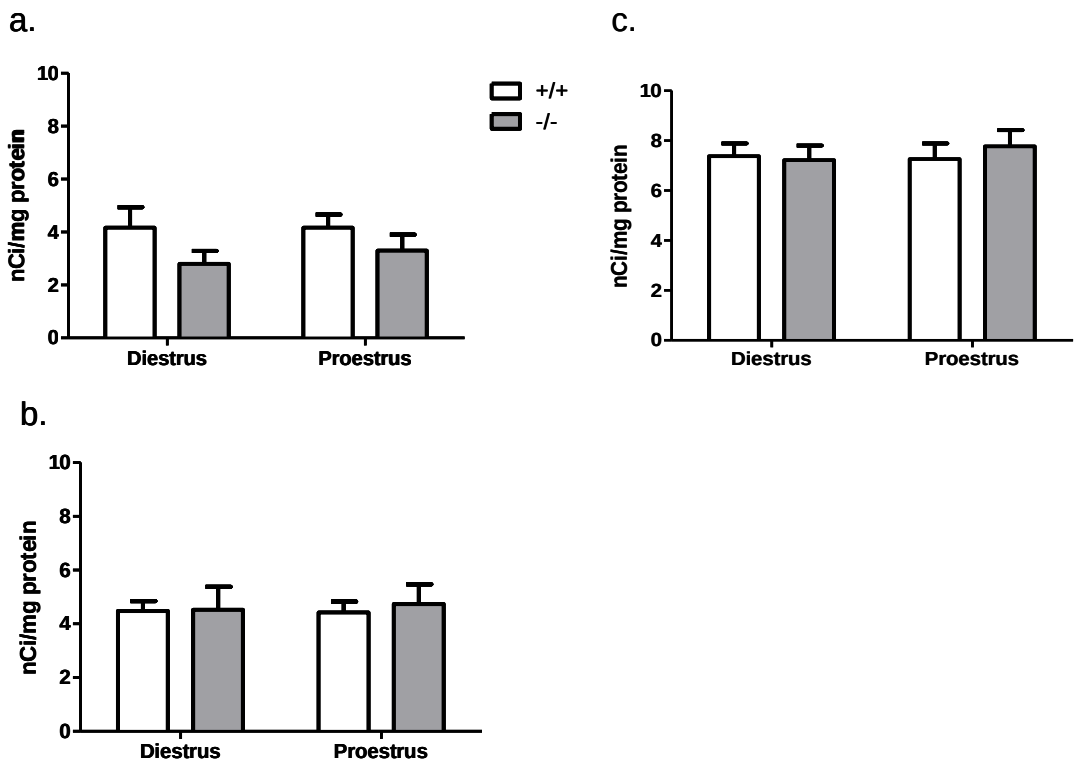

Fig. 4-4. D2-like binding in the (a) NAcc shell (b) NAcc core and (c) CP in female NT+/+ and NT-/- mice. Data are expressed as mean binding density (nCi/mg protein) ± S.E.M. D2-like binding was not significantly different in any of the brain regions investigated.

CHAPTER 5: GENERAL DISCUSSION

The studies described in this thesis sought to interrogate the function and tone of the mesocorticolimbic and nigrostriatal DA systems in mice deficient in NT. The results indicate that lack of NT during development results in sex-specific DA system plasticity, particularly, changes in DA receptor expression and function. These results suggest a regulatory role for NT during the development of the DA systems. In addition, these studies implicate disrupted NT neurotransmission in the neurobiology of disorders associated with DA system abnormalities, specifically schizophrenia. These studies also raise several interesting questions which are discussed below. In addition, future studies utilizing NT-/- mice are suggested.

5.1 THE ROLE OF NT IN NEURAL DEVELOPMENT

Constitutive gene knockout often results in compensatory alterations during development. Examining developmental modifications in gene knockout mice can be utilized as an opportunity to explore the role of the targeted gene in developmental processes. This approach is especially valuable to gain insight into diseases involving developmental disruptions, such as schizophrenia. This dissertation specifically sought to investigate developmental modifications in the mesolimbic and nigrostriatal DA systems as a consequence of NT gene knockout.

Striatal and Cortical Development

The studies presented in this thesis, and several other studies summarized in Chapter 1 (for review also see Binder et al. (2001b)) show NT plays an important role in the maintenance of DA system homeostatis. In the case of NT deficiency during development as a result of NT gene knockout in mice, DA receptor function and expression are perturbed. These findings support a role for NT in regulating DA system development. Indeed, some studies suggest NT regulates neural development. In neocortical regions and in the anterior CP in rats, NT and NTRs are expressed in high

amounts transiently in the prenatal and early postnatal period, followed by a rapid decline to almost undetectible levels (Sato *et al*, 1992; Sato *et al*, 1991; Sato *et al*, 1990). This transient peak and decline in expression occurs before the maturation of a neuronal network, and suggests NT might serve a regulatory role in cortical and striatal development that is distinct from its physiological role during adulthood. Transient high levels of NTRs are also observed in the brains of human infants followed by a decline at around 1 year of age (Zsürger *et al*, 1992). Finally, NT promotes dendrite elongation and dendritic spine maturation in rat cerebral cortex *in vitro*, possibly through action at NTS1 and NTS3 receptors, although the exact mechanism is not understood (Gandou *et al*, 2010). From these studies and the experiments presented in this dissertation, it is clear that future experiments are needed to characterize the role of NT in the neural development of cortical and striatal regions.

The studies in this dissertation focused on characterizing the changes in DAergic tone in striatal and cortical regions in NT deficient mice, but, given the possible role of NT in neuronal growth, future studies might scrutinize these brain regions in NT-/- mice for possible anatomical changes or altered connectivity in mesocorticolimbic or nigrostriatal circuits. NT-/- mice do not show any gross neuroanatomical changes, but future studies might address the possibility of more subtle anatomical alterations, such as changes in regional connectivity in mesocorticolimbic or nigrostriatal circuitry or changes in dendritic maturation in neurons in these regions. As presented in Chapter 3, the DOPAC/DA ratio in the NAcc compared to the FCTX was increased in NT-/- mice compared to NT+/+ mice, suggesting there may be an increase in DA utilization in the NAcc relative to the FCTX in NT-/- mice compared to NT+/+ mice (Table 3-1). This result may reflect subtle changes in corticostriatal neurocircuitry in NT-/- mice that could be investigated using anatomical methods or diffusion tensor imaging techniques.

Developmental Timeline

Future studies might also assess the timeline for development of the observed alterations in DA receptor densities in NT-/- mice. Increases in striatal D2-like binding densities were observed in adult male NT-/- mice, while decreased striatal D1-like binding densities were found in adult female NT-/- mice. Like NTR expression, DA receptor expression also undergoes transient fluctuations during development in rats and humans (Andersen *et al*, 2000; Seeman *et al*, 1987a). Interestingly, in rats, this process is sexually dimorphic; in the NAcc and dorsal striatum, male rats show an overproduction of D1 and D2 receptors which peaks during puberty followed by a subsequent pruning of these receptors which plateaus during adulthood (Andersen *et al*, 2000). In contrast, female rats show little overproduction and pruning of DA receptors compared to males. Alterations in DA receptor pruning during adolescence has been theorized to be involved in the etiology of developmental disorders that show sex differences in severity and timecourse, such as schizophrenia (Andersen *et al*, 2000). It has not been determined whether mice undergo a similar process in DA receptor development, but future studies might survey DA receptor densities at several developmental timepoints in both wildtype mice and NT-/- mice to determine when differences in DA receptor densities first occur. If a specific age of onset of the DA receptor alterations is determined, follow-up studies might utilize conditional knockout of NT to determine if there is a critical period in which NT is required for normal expression of DA receptors to occur. These future studies are outlined below.

Proposed Future Experiments

Specific Aim #1: Characterization of DA and NT system development in male and female mice. Rats undergo transient fluctuations in striatal and cortical expression of NT, NTRs, and DA receptors during development. These processes are thought to underly the maturation of the adult neuronal network. Fluctuations in DA receptor

expression in rat brains during development were shown to be sexually dimorphic. The proposed studies seek to investigate whether a similar developmental process occurs in mice, and whether this process is sexually dimorphic. *Methods:* Male and female mice will be sacrificed at several developmental timepoints, including infancy (postnatal days 1 and 7), weaning/adolescence (postnatal day 14 and 21), young adulthood (postnatal days 28, 40), adulthood (postnatal days 60 and 90), and old age/reproductive senescence (postnatal days 270 and 365). NT, NTRs, and DA receptor expression and binding densities will be measured at each of these timepoints in various brain regions including: cortex, striatum, SN, VTA, hypothalamus, and hippocampus. It is hypothesized that significant fluctuations in NT and DA systems may occur during adolescence, young adulthood, and old age, and these fluctuations may be sexually dimorphic. These results will be utilized to pinpoint a developmental timeline for Aim #2.

Specific Aim #2: Does NT regulate the development of cortical and striatal neural maturation and circuitry? The studies presented in this thesis indicate NT regulates striatal DA receptor function and expression. As mentioned above, an *in vitro* study suggests NT plays a broader developmental role in regulating dendritic outgrowth and maturation, possibly through action at NTS1 and NTS3 receptors (Gandou *et al*, 2010). The proposed studies seek to verify this *in vivo*. Specifically, we will ask whether NT, NTS1, and NTS3 are necessary for the neural development of cortical and striatal regions and for development of the mesocorticolimbic circuit. *Methods:* Male and female NT-/- mice as well as mice lacking the NTS1 gene (NTS1-/-) and mice lacking the NTS3 gene (NTS3-/-) will be utilized. Using microscopy techniques, dendritic morphology will be measured in cortical and striatal regions in the knockout mice and compared to wildtype mice. It is predicted that NT-/-, NTS1-/-, or NTS3-/- mice may show dimished dendritic elongation and dendritic spine maturation compared to wildtype mice. In addition, based on the developmental timeline obtained in Aim #1, DA receptor

expression and binding will be measured in knockout and wildtype mice at selected developmental timepoints and at adulthood. It is predicted that male NT-/- mice may show diminished pruning of D2 receptors compared to wildtype mice, and that this may underly the elevation in D2 receptors observed in adult NT-/- mice in the studies presented in this thesis. Finally, circuit connectivity within the mesocorticolimbic DA systems of knockout and wildtype mice will be measured using either immunohistochemical methods or diffusion tensor imaging. It is predicted that NT-/- mice, and perhaps NTS1-/- or NTS3-/- mice, may show alterations in VTA-accumbens, cortico-accumbens, or striatopallidal projections compared to wildtype mice.

5.2 NT KNOCKOUT, DA RECEPTOR ALTERATIONS, AND BEHAVIORAL CHANGES

While investigating developmental modifications in gene knockout mice can be useful for determining the role of the targeted gene in developmental processes, compensatory alterations during development may limit the general conclusions that can be made about the physiological role of the gene of interest. Particularly, one might ask, is the observed behavioral phenotype in the knockout mouse due to knockout of the gene of interest or is it due to developmental modifications? Attributing a loss of function to just one particular gene or physiological variable may be too simplistic, as several variables likely contribute to the loss of function in a complex biological system (Deutscher *et al*, 2008). Instead, it might be concluded that knockout of a single gene is likely a major contributing factor in the observed behavioral alterations, although it may work synergistically with other factors to produce this phenotype. Regarding the studies presented in this dissertation studies, does lack of NT primarily cause the behavioral alterations observed in male NT-/- mice or are these behaviors caused by the developmental modifications observed in NT-/- mice (i.e., increased striatal D2 densities)? The probable causal factor for the behavioral phenotype might be identified

by comparing the results obtained in NT-/- mice with results from studies utilizing pharmacological blockade of NTRs in rats. Systemically administered NTR antagonists quickly bind NTRs, blocking NT neurotransmission in rats, and produce behavioral effects within 1 hour of administration (Cáceda *et al*, 2012). Given their rapid effects, the behavioral consequences of NTR antagonists are very unlikely to be due to changes in DA receptor expression and are most likely due to acute blockade of NTRs and NT neurotransmission.

In the studies in this dissertation, male NT-/- mice showed diminished locomotor response to acute amphetamine (at the 2 mg/kg) and diminished sensitization to amphetamine (at the 1 mg/kg dose) (Chapter 2). In contrast, NTR antagonist administration does not affect the acute hyperlocomotor effects of amphetamine (Cáceda *et al*, 2012; Casti *et al*, 2004; Panayi *et al*, 2002). The disparity between these results suggest the diminished locomotor response to acute amphetamine observed in NT-/- mice is not due to lack of NT, but is due primarily to developmental changes as a consequence of NT gene knockout, possibly increased striatal D2 receptor densities. In line with this reasoning, striatopallidal D2 receptor-expressing neurons have an inhibitory effect on the locomotor effects of amphetamine (Durieux *et al*, 2009). Thus an increase in striatal D2 receptors in NT-/- mice may attenuate the acute hyperlocomotor effects of amphetamine. The observation that female NT-/- mice do not show increased D2 receptors and also do not show diminished locomotor response to amphetamine supports the hypothesis that behavioral alterations observed in male NT-/- mice are due to increases in striatal D2 receptors. In contrast, like NT gene knockout in mice, NTR antagonist administration in rats diminishes behavioral sensitization to amphetamine (Costa *et al*, 2007; Costa *et al*, 2001; Panayi *et al*, 2002). Thus, it can be concluded that the diminished amphetamine sensitization observed in male NT-/- mice is probably primarily due to lack of NT and not developmental compensations.

In a previous study (Kinkead *et al*, 2005), male NT-/- mice showed increased startle response anddecreased PPI, although these behavioral alterations were not observed in the experiments presented in this thesis (discussed below). Male NT-/- mice also showed diminished effects of amphetamine on PPI (Kinkead *et al*, 2005). As NTR antagonist administration does not affect baseline startle amplitude orPPI, it might be concluded that decreased PPI and increased startle amplitude in NT-/- mice were due primarily to developmental alterations in NT-/- mice. D2 receptors are known to modulate PPI in mice (Ralph-Williams *et al*, 2002b), and thus the change in striatal D2 receptor expression in NT-/- mice could likely disrupt PPI. In line with this reasoning, female NT-/- do not show PPI deficits (Kinkead *et al*, 2005) nor do they show altered striatal D2 densities (Chapter 4). In contrast, NTR antagonist administration, like NT gene knockout, diminishes PPI disruption by amphetamine (Cáceda *et al*, 2012), so it might be concluded that the diminished effect of amphetamine on PPI seen in male NT-/- mice is due primarily to lack of NT and not developmental compensations.

5.3 NT DEFICIENT MICE AS AN ANIMAL MODEL OF SCHIZOPHRENIA?

As detailed in the Introduction, in clinical studies, a subset of schizophrenic patients showed deficits in NT concentrations in CSF compared to nonschizophrenic subjects (Breslin *et al*, 1994; Lindström *et al*, 1988; Manberg *et al*, 1985; Nemeroff *et al*, 1989b; Sharma *et al*, 1994; Widerlöv *et al*, 1982), and a deficit in NT was associated with increased symptom severity (Garver *et al*, 1991; Sharma *et al*, 1997). For this reason, mice deficient in NT were evaluated for behavioral and physiological alterations related to those observed in schizophrenics. Increased D2-type receptor binding in striatal regions has been consistently shown in schizophrenic patients (Seeman, 1987; Seeman *et al*, 2000; Wong *et al*, 1997). Likewise, NT-/- mice show increased striatal and accumbal D2-type receptor expression and binding density. In addition, in dozens

of mouse models, knockout of various genes encoding proteins noted to be involved in schizophrenia pathophysiology results in increased striatal D2-type receptor binding (for review see Seeman et al. (2006)). Developmental and pharmacological animal models of schizophrenia all produce increases in striatal D2-type binding, including isolation rearing paradigms, neonatal ventral hippocampal lesions, and repeated administration of psychostimulants (Seeman, 2011). This phenomenon is thought to be indicative of plastic changes in DA system functioning and sensitivity, which is theorized to underly many of the symptoms of schizophrenia (Grace, 1991; Seeman, 2011). From the studies in this dissertation, it is clear that NT gene knockout is sufficient to produce elevated striatal D2-type binding densities, suggesting NT deficiency is a valid mouse model of schizophrenia. In addition, these results suggest a deficiency in NT may contribute to the development of schizophrenia.

Some schizophrenic patients show supersensitivity to indirect DA agonists, and when administered these drugs in low doses, psychotic symptoms are exacerbated (for a meta-analysis see (Lieberman *et al*, 1987)). However, some schizophrenics show no response to DA agonists, and there is evidence that DA agonists might improve negative symptoms in some schizophrenic patients (Lindenmayer *et al*, 2013; Sanfilipo *et al*, 1996). Most animal models relevant to schizophrenia also show supersensitivity to direct and indirect DA agonists, as measured by the effects of these drugs on locomotor activity and sensorimotor gating, while a few show subsensitivity to DA agonists (Seeman, 2011). NT-/- mice fall in the latter category, as they show subsensitivity to the effects of the indirect DA agonist amphetamine and the selective D1-like agonist SKF-82958 on locomotor activity and PPI (Chapter 2). These findings suggest NT-/- mice may have potential as an animal model of negative schizophrenia-like symptoms. In line with this reasoning, some clinical studies have shown the decreased CSF NT levels observed in some schizophrenics are associated with negative symptoms (Garver *et al*,

1991; Sharma *et al*, 1997). Historically, most animal studies have emphasized modeling positive symptoms of schizophrenia while neglecting negative symptoms, and negative symptoms are notoriously difficult to treat in schizophrenic patients (Foussias and Remington, 2010; Hanson *et al*, 2010). For this reason, future studies might examine NT-/- mice for behaviors relevant to the negative symptoms of schizophrenia, including anhedonia, motivation, and social behavior.

Sensorimotor gating deficits are thought to underly positive symptoms in schizophrenia, and disruptions in PPI have been reliably observed in schizophrenic patients (Braff *et al*, 2001; Kumari *et al*, 2000; Parwani *et al*, 2000). Deficits in PPI are also consistently observed in developmental, pharmacological, and genetic animal models of schizophrenia (Geyer *et al*, 2001; Powell *et al*, 2009). As mentioned above, male NT-/- mice were previously shown to have significantly decreased PPI, a measure of sensorimotor gating, compared to NT+/+ (Kinkead *et al*, 2005). However, the experiments presented in this thesis failed to replicate these PPI deficits. As noted, the reason for this might be due to the smaller sample sizes utilized in the present studies, or to uncontrolled environmental factors. Lack of replication of previously observed PPI deficits have been observed in other mouse models of schizophrenia and were attributed to minor differences in the laboratory environment and animal housing (Karl *et al*, 2011).

One environmental factor that may alter PPI is stress (Bakshi *et al*, 2012; Grillon and Davis, 1997). It is possible that differences in sensitivity to environmental stressors in NT-/- and NT+/+ mice may play a role in modulating PPI disruption in these animals. In line with this theory, compared to NT+/+ mice, both male and female NT-/- mice were previously shown to have increased startle response to an acoustic stimulus, an indicator of fear and anxiety (Davis, 2006; Kinkead *et al*, 2005). In addition, compared to NT+/+ mice, female NT-/- mice showed increased marble burying, an anxiety-like behavior in rodents (unpublished results). Together, these data suggest mice deficient

in NT may be more vulnerable to anxiety. This hypothesis could be tested directly in future experiments proposed below. These follow-up studies would be especially pertinent to understanding schizophrenia etiology, as stress often precipitates psychotic episodes in people with schizophrenia (Corcoran *et al*, 2003). Utilizing NT-/- mice and established rodent behavioral protocols for inducing stress and measuring stress response, we can explore whether NT system disruption interacts with environmental stress to produce behavioral and physiological disruptions relevant to schizophrenia.

Proposed Future Experiments

Specific Aim #1: The effects of NT deficiency on sensitivity to reward and social behavior. In schizophrenic patients, deficits in CSF concentrations of NT in schizophrenics were associated with more severe negative symptoms (Garver *et al*, 1991; Sharma *et al*, 1997). Mice lacking NT show subsensitivity to amphetamine, suggesting a lack of NT may be associated with decreased responsivity to reward, a rodent behavior relevant to anhedonia, a negative symptom of schizophrenia. For this reason behaviors relevant to other negative symptoms of schizophrenia including reward sensitivity, motivation, and social behavior will be measured in male and female NT-/- mice compared to NT+/+ mice. Methods: Anhedonia will be measured in NT-/- and NT+/+ mice using the sugar water preference test. Reward sensitivity will be measured using the conditioned place preference test. Social and aggressive behaviors will also be measured in knockout and wildtype mice, utilizing social preference and memory tests and the home cage intruder challenge test. It is hypothesized that NT-/- mice may show decreased sugar water preference, decreased conditioned place preference for amphetamine, decreased social behavior, and more aggressive behavior compared to NT+/+ mice.

Specific Aim #2: The effects of NT deficiency on behavioral and endocrine response to stress. Several studies suggest NT may play a role in regulating

behavioral and endocrine response to stress. The hypothalamic-pituitary-adrenal (HPA) axis regulates endocrine response to stress. Studies utilizing NT receptor antagonists suggest endogenous NT does not modulate baseline HPA axis function but mediates HPA axis response to restraint stress (Geisler *et al*, 2006). Studies in our lab indicate mice lacking NT may have increased anxiety-like behaviors, specifically, increased acoustic startle response (Kinkead *et al*, 2005) and increased marble burying behavior (unpublished results). These data suggest mice deficient in NT may be more vulnerable to stress. These studies aim to characterize behavioral and endocrine response to stress in NT-/- mice compared to NT+/+ mice. The results of these studies will further elucidate the role of endogenous NT in regulating HPA axis function. In addition, these studies will evaluate whether dysfunction in NT neurotransmission interacts with environmental stressors to produce behavioral and endocrine states relevant to psychiatric disorders. *Methods:* Baseline HPA axis function (plasma corticosterone and adrenocorticotropic hormone (ACTH) levels, hypothalamic corticotropin-releasing factor (CRF) expression) and behaviors relevant to anxiety (open field behavior, elevated plus maze behavior) will be evaluated in NT-/- mice compared to NT+/+ mice. Behavioral and HPA axis response to restraint stress and forced swim stress will also be evaluated in knockouts and wildtypes. It is hypothesized that NT-/- mice may show increased behavioral and endocrine response to stress.

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