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Probing the Mesolimbic Dopamine Reward System: A Common Neurocircuitry in Substance Abuse and Schizophrenia

A Dissertation Presented

by

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Abstract of the Dissertation

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Substance abuse is more prevalent among patients with schizophrenia than in the general population. Interestingly, the pathophysiology of schizophrenia is thought to share many overlapping features with drug addiction, including disruption of the mesolimbic dopaminergic pathway. My thesis work was aimed at investigating drug-induced alterations in dopamine function that leads to addiction-related behavior using animal models of substance abuse. In the first part of my studies, I combined small animal imaging with behavioral tests to probe the effects of abused inhalants on overall brain activity and the dopaminergic system in rats. These studies lead me to the second part of my thesis, where I characterized a dopamine-based phenotype in mice that have targeted deletion of a schizophrenia susceptibility gene, *NRG1*, to investigate the contribution of NRG1 signaling on dopamine dysfunction in schizophrenia. First, a radiolabeled analog of glucose [(18)FDG] monitored regional changes in glucose uptake during a drug-preference behavioral task. Next, striatal D2 dopamine receptor density was measured by [(11)C]raclopride, a D2 receptor antagonist. In another set of studies, [(11)C]raclopride was used as an assay for synaptic dopamine release upon drug challenge. In parallel, baseline and drug effects on synaptic dopamine release and metabolism was measured *in vivo* using microdialysis. The results of the first part of my thesis indicated that the rewarding effects of toluene are mediated, at least in part, by the DA system. Further, in the second part of my thesis, I investigated whether genetic disruption of a candidate schizophrenia susceptibility gene, *NRG1*, altered drug seeking behavior and / or the DA system. My results suggest that mice heterozygous for a disruption of the Type III

isoforms of the *Nrg1* gene are more responsive to the behavioral and neurochemical effects of cocaine. Additional results demonstrate that *Nrg1* heterozygous mice have alterations in striatal dopamine receptor levels and altered baseline DA metabolism. These results mirror clinical reports demonstrating DA sensitization in neuroleptic treated schizophrenic patients. My results validate the use of behavior and small animal imaging to study drug abuse, and therefore, this paves the way for future studies on other drugs of abuse or in other genetic animal models.

DEDICATION

I dedicate this thesis to My Rock, My Foundation.

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List of Abbreviations

Abbreviations and Acronyms

AMPT	**α-methly-para-tyrosine**
APA	**American Psychiatry Association**
ATP	**adenosine-5'-triphosphate**
BP	**3,4-dihydroxyphenylacetic acid**
cAMP	**cyclic adenosine monophosphate**
CB	**cerebellum**
CNS	**central nervous system**
COMT	**catechol-O-methyltransferase**
CPP	**conditioned place preference**
DA	**dopamine**
DAT	**dopamine transporter**
DOPAC	**3,4-dihydroxyphenylacetic acid**
DVR	**distribution volume ratio**
FBP	**filtered backprojection**
(18)FDG	**(18)F-fluorodeoxyglucose**
FWHM	**full width half maximum**
G-protein	**guanosine nucleotide-binding protein**
HVA	**homovanillic acid**
ICD	**intracellular domain**
KO	**knockout**
MAOs	**monoamine oxidase inhibitors**
MAP	**maximum a posteriori**
microPET	**small animal PET**
MRI	**magnetic resonance imaging**
NAc	**nucleus accumbens**
nAChRs	**nicotinic acetylcholine receptors**
NMDA	**N-methyl-D-aspartate**
NRG1	**Neuregulin 1 human gene**
Nrg1	**Neuregulin 1 mouse gene**
NRG1	**Neuregulin 1 protein**
PET	**positron emission tomography**
PFC	**prefrontal cortex**
PPI	**prepulse inhibition**
ROI	**region of interest**
SNPs	**single nucleotide polymorphism**
SPM	**statistical parametric mapping**
STR	**striatum**
VTA	**ventral tegmental area**

Acknowledgements

After my bachelor's degree in Media Arts from Hunter College, I decided that my real passion was in medical science, so, I eagerly signed up for a few basic science courses at Stony Brook University. Quite honestly, I never imagined, sitting in that crowded auditorium with undergraduates during the first semester freshman chemistry class, that one day I would be sitting here in my laboratory writing the Acknowledgement page for my Doctoral Dissertation. Actually, it was in that same chemistry course, back in 2003, where I was first introduced to Dr. Joanna Fowler and her research group at Brookhaven National Laboratory. During her lecture, she taught that addiction, specifically drug abuse, was a biologically based brain disorder which piqued my naïve curiosity. I guess you can say I got "hooked" on addiction research. Later, I became aware that they were doing pioneering brain imaging work on humans and animals. Thus, the journey began, driving to and fro Stony Brook University to Brookhaven National Laboratory.

First, I would like to thank my mentors, colleagues, and friends at BNL: Joanna Fowler for taking a chance with, a young, naïve, and inexperienced me, my first mentor at BNL, Madina Gerasimov, who taught me how fulfilling research can be, to Andrew Gifford, for his curiosity, to Stephen Dewey for teaching me not to give up, no matter how difficult the task, to Wynne Schiffer, for teaching me that research can be accessible and fun, and to Dave Alexoff, the jack of all trades.

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CHAPTER 1

Introduction

In the 18th and 19th centuries, witchcraft and demonic possessions were common explanations for mental illnesses, thus, treatments incorporating rituals of atonement and purification were commonly practiced by persons knowledgeable in medicine. At the turn of the century, pioneering researchers, psychiatrists, and scholars like Benjamin Rush advocated for the humane treatment of the mentally ill. He and his colleagues published the first American textbook of psychiatry entitled "*Medical Inquiries and Observations upon Diseases of the Mind*" (1812). Other psychiatrist, notably the Swiss born Adolf Meyer, embraced mental health research which made possible the first inpatient facility with twelve beds in the United States for the mentally ill in 1912 (Burnham, 1967). It was not until half a century later that antipsychotics revolutionized the treatment of psychosis. The first antipsychotics were D2 dopamine receptor antagonist, based on the hypothesis that hyperdopaminergic function was the key dysfunction of psychosis (Carlsson, 1987; Seeman et al., 1976). The dopamine theory was based largely on the observation that the dopamine releasing drug, amphetamine, induces a schizophrenia-like state in non-psychotic subjects while exacerbating psychoses in schizophrenic patients. Since then, a great deal of research emphasizing the mechanism of dopamine dysfunction in the pathophysiology of both substance abuse and schizophrenia has been achieved.

This introductory chapter is divided into three sections. The first describes the mesolimbic dopamine reward pathway with an emphasis on dopamine transmission from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), a critical circuit for

drug reinforcement and reward. In conjunction, I will detail a common mechanism by which drugs of abuse alter the dopamine reward pathway. The second section is aimed at understanding the rewarding effects of solvent abuse. Here, a special focus will be made to introduce neurobehavioral animal models of drug abuse. Lastly, I present an animal model of substance abuse and schizophrenia comorbidity to understand the impact of genetic predisposition on drug dependence in the schizophrenic population.

Mesolimbic dopamine reward circuit

Dopamine tracts in the brain

The dopamine system is highly organized structurally and is broadly classified into two groups: (1) meso(nigro)striatal system, and (2) mesolimbic and mesocortical systems (Fig 1.1). The mesostriatal system projects from the substania niagra to the striatum and is highly involved in motivation and voluntary movement. The mesolimbic and mesocortical systems project from the VTA to the limbic and frontal cortical regions, respectively. These regions participate in reward reinforcement learning, one's response to internal and external stimuli (positive or negative), and cognition.

The mesolimbic dopamine circuit is the major site of dopamine neurons which begin at the VTA. The dopamine neurons in the VTA have far reaching projections to the limbic forebrain – NAc, amygdala, hippocampus, and prefrontal cortex (PFC). Of these, the VTA-NAc pathway is the most important mesolimbic dopamine projection for reinforcement and reward-driven behaviors. Therefore, the VTA is the major site of action of most drugs of abuse. Drugs of abuse increase the level of dopamine in the

neurons of the VTA, thus activating the same VTA-NAc pathway critical for natural reward and motivation (Fiorino et al., 1993; Di Chiara and Imperato, 1988). Studies in animals have demonstrated that dopamine neurons increase their firing rate when the animals are presented with reward-associated stimuli requiring behavior responses (Grace and Floresco, 2007). Thus, the effectiveness of the drugs to sustain drug-seeking behavior in animals is highly correlated with the abuse potential of the drug in humans (Volkow et al., 1996).

Additionally, it is thought that the limbic system may be involved in the positive symptoms of schizophrenia (Paulson and Robinson, 1996). It has been proposed that hyperdopaminergic function is the underlying cause of psychoses in schizophrenic patients, thus, the mesolimbic circuits are a main target for antipsychotic drugs (Carlsson, 1987).

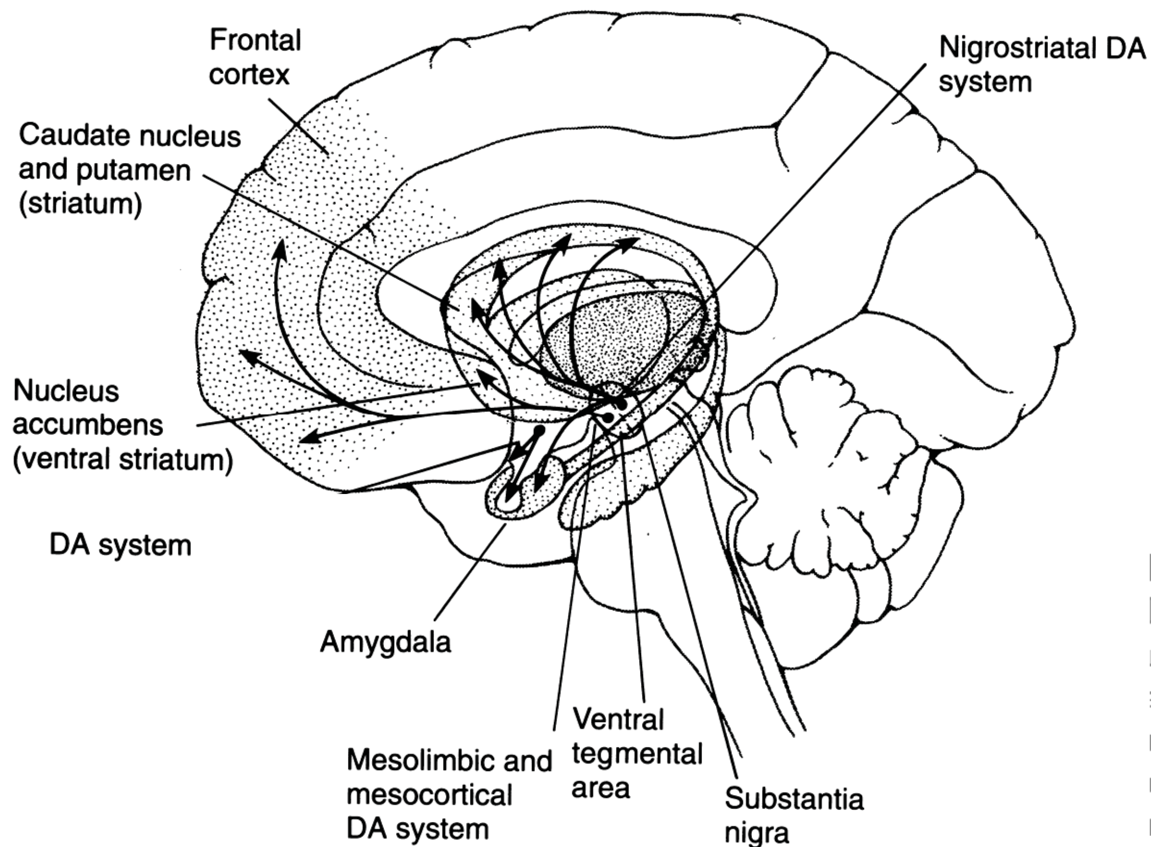


Figure 1.1 A midsagittal section showing dopamine tracts in the human brain: The neurons in the mesolimbic and mesocortical pathways begin at the ventral tegmental area and projections to the limbic forebrain, in particular the nucleus accumbens (ventral striatum) important for reward, amygdala involved in reinforcement learning, and prefrontal cortex critical for cognition. The nigrostriatal system runs parallel to the mesolimbic pathway. Its projections stem from the substantia nigra to the caudate nucleus and putamen (striatum). Perturbations in the neurons in these circuits are implicated in many brain diseases, like Parkinson's, addiction, and schizophrenia. (adapted from Kandel et al., 2000)

Dopamine transmission

The general scheme of dopamine transmission (Fig. 1.2) divides the processes into four steps, (1) synthesis of transmitter, (2) storage into vesicles and release into synaptic cleft, (3) interaction with receptors on the postsynaptic membrane, and (4) removal / degradation of transmitter from the synaptic cleft. Perturbations in any one or combinations of these processes have been characterized as hallmarks of brain disorders, including Parkinson’s and schizophrenia [for review, see (Cummings, 1992)].

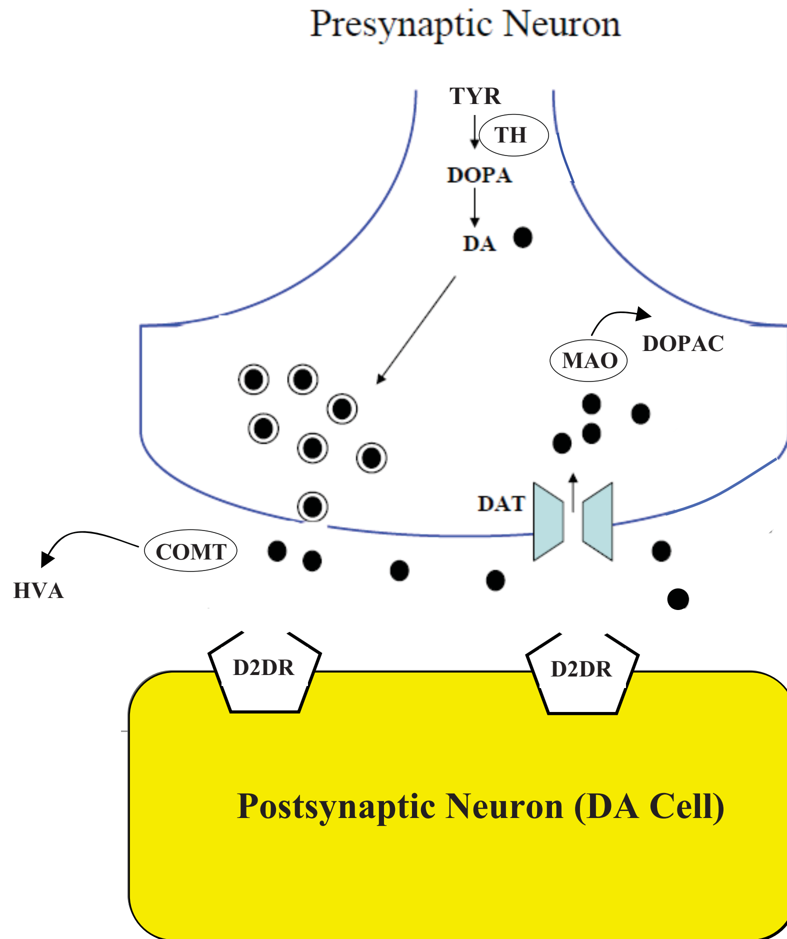


Figure 1.2 Dopamine nerve terminal – shown is a simplified model showing key steps in the synthesis and degradation of dopamine. Dopamine (DA) is synthesized from tyrosine (TYR). The enzymatic activity of tyrosine hydroxylase (TH) converts TYR to L3,4-hydroxyphenylalaine (DOPA), the immediate precursor to DA. DA is stored in vesicles. Upon cell firing, vesicles release DA into the synaptic cleft. Once in the extracellular space, the neurotransmitter binds to its target protein, D2 dopamine receptor, or recycled back up the presynapse, a process termed DA reuptake, by dopamine transporters (DAT). DA degredation enzymes are monoamine oxidase (MAOs), intracellular, and catechol-O-methyltransferase (COMT) in the cytoplasm.

Catecholamine transmitters

Dopamine is a small-molecule transmitter identified as a catecholamine whose chemical structure contains a catechol nucleus, a 3,4-dihydroxylated benzene ring. The catecholamine transmitters – dopamine, norepinephrine, and adrenaline (epinephrine) are all synthesized from the amino acid tyrosine in the same biosynthetic pathway containing four enzymes: tyrosine hydroxylase, aromatic amino acid decarboxylase, dopamine β-hydroxylase, and phenylethanolamine-*N*-methyltransferase (Fig. 1.3). Norepinephrine is used as a transmitter in the CNS by cell bodies located in the locus cereleus, a nucleus of the brain stem with many modulatory functions. In the PNS, norepinephrine is the transmitter in the postganglonic neurons of the sympathetic nervous system. Similarly, epinephrine, produced by the adrenal gland, binds to adrenergic receptors to regulate various processes of metabolism.

Disturbances of neurotransmitter metabolism are hallmarks of many brain disorders (Cohen and Carlezon, 2007; Koob, 1992; Schultz et al., 1997). For example, Parkinson's disease is defined by the deficiency in dopamine in the basal ganglia, an area of the brain that contains 80 % of brain dopamine and makes up less than 0.5 % of the brain (Carlsson, 1959). Diseases of the basal ganglia characteristically produce involuntary movements, including tremors and dystonia. Remarkably, treatment with an amino acid, L-3,4-hydroxyphenylalaine (L-DOPA), a precursor to dopamine, ameliorates symptoms of Parkinson's disease [for review, see (Cummings, 1992)]. Another pathophysiology of Parkinson's is a marked decrease in dopaminergic projections from the substantia nigra to the basal ganglia. However, how those few healthy dopamine neurons carry out the entire function of the substantia nigra system remains unknown.

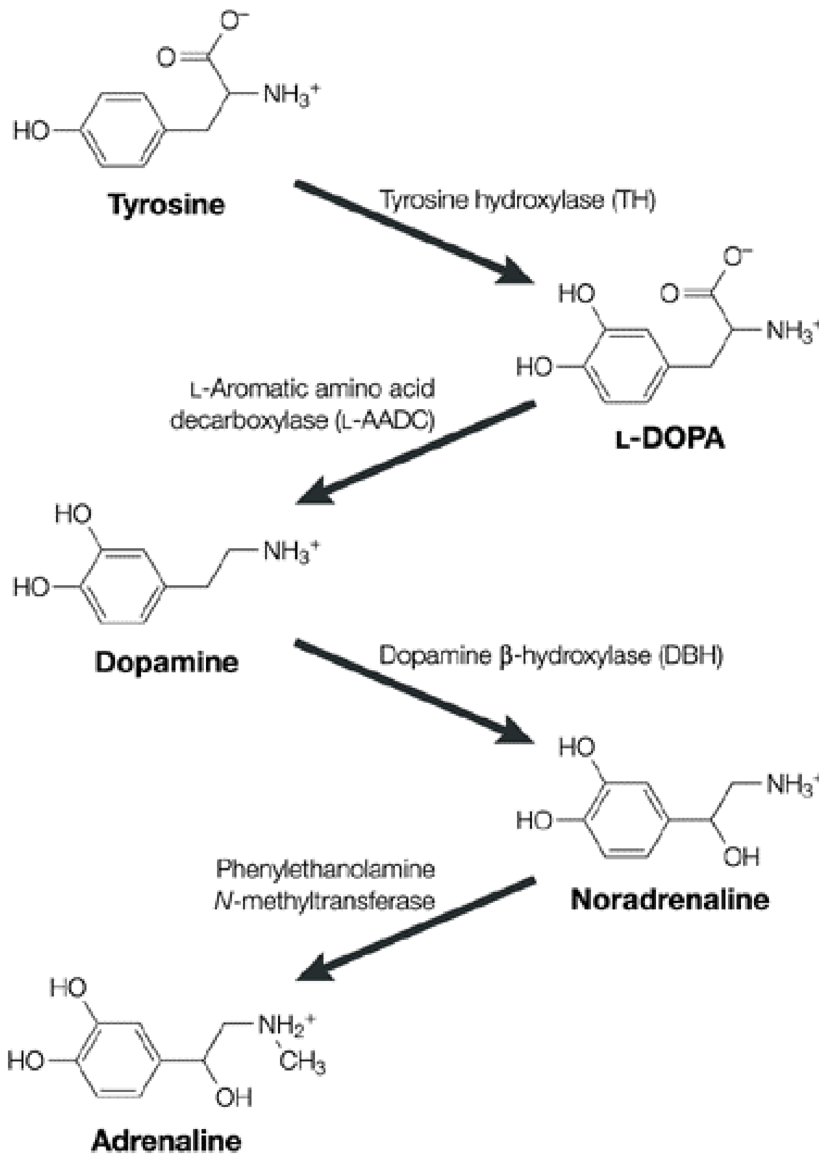


Figure 1.3 Biosynthesis of catecholamine transmitters – catecholamine transmitters include dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline). They are small-molecule transmitters identified as a catecholamine due to its chemical structure containing a catechol nucleus, a 3,4-dihydroxylated benzene ring. All three transmitters are synthesized from the amino acid tyrosine and L-DOPA. (adapted from Kandel et al., 2000)

Presynaptic transmitting process and recycling

Synaptic vesicles are filled with the chemical transmitter dopamine. In response to an action potential, calcium (Ca2+) permeability is increased, and the vesicles fuse with the cytoplasmic membrane. The fusion causes vesicular release of the transmitter at the nerve terminals, which then travels a small distance (~ 3.5 nm) to its target postsynaptic receptor. Reuptake of dopamine from the synaptic cleft is the most common mechanism for degradation. The nerve terminals express dopamine transporter (DAT) molecules that mediate the removal of dopamine which terminate synaptic transmission. There are other degradation enzymes that regulate the concentration of dopamine but are not specifically involved in terminating synaptic transmission. For example, monoamine oxidase (MAOs) regulates the intracellular concentration of dopamine. The inhibition of MAOs is a common mechanism for antidepressants. In the cytoplasm, catechol-O-methyltransferase (COMT), and the concentration of its metabolite, homovanillic acid (HVA), in body fluids serves as an indirect diagnostic indication of the efficacy of drugs that affect the synthesis or degradation of the enzyme (Davis et al., 1991).

Postsynaptic receptive process

Dopamine receptors are G-protein-coupled receptors (GPCR). Recognition of the neurotransmitter and activation of effectors are carried out by distinct and separate molecules. The receptor molecule is coupled to its effector molecule by a guanosine nucleotide-binding protein (G-protein). The effector is an enzyme that produces a second messenger, e.g. cAMP and inositol polyphosphate, which in turn trigger a biochemical cascade.

At least five major types of dopamine receptors are thought to exist (D1, D2, D3, D4, and D5). The D1 and D5 dopamine receptors are coupled to the G-protein (Gs) that activates adenylyl cyclase, the enzyme that converts ATP to cAMP, and highly expressed in neurons of the cortex and hippocampus. The D2 dopamine receptors are coupled to inhibitory G-protein (Gi) that inhibits adenylyl cyclase. The D2 receptors are highly expressed in neurons of the caudate and the limbic systems, specifically in the NAc which is the major site of DA release. The D3 and D4 receptor expression are restricted to the cortex and limbic region, but have low expression in the basal ganglia.

Drugs of abuse

Drug dependence is characterized in both the *Diagnostic and Statistical Manual of Mental Disorders* [DSM-IV; American Psychiatry Association (APA, 1994) and the International Classification of Diseases (ICD-10; World Health Organization, 1992) as drug-seeking behavior involving compulsive use of high doses of drugs for no clear medication indication.

Several types of alterations in brain function have been described in the addiction field (Koob and Simon, 2009; Koob and Le Moal, 2005). Tolerance and sensitization are relatively simple manifestations of learning and memory that refer to decreases and increases in the strength of a response to a stimulus induced by past experiences with the same or related stimuli (Kauer and Malenka, 2007; Adcock et al., 2006). Tolerance is a decreased response to continued use of the drug which is a natural cellular adaptation for the neural circuit. Unfortunately, the consequence being that the drug abuser takes increasing amounts of the drug to achieve the same high leading to overdose and death.

The time it takes to reach tolerance depends on the drug and the individual's innate tolerance level. Physical dependence (addiction) gives rise to a physical withdrawal syndrome upon cessation of drug exposure, and may contribute to the aversive state and high rates of relapse seen during stages of withdrawal (Nestler and Aghajanian, 1997).

A common mechanism of action

Some of the most powerful drugs are those that mimic endogenous dopamine, thus, activating the same neuronal circuits as natural rewards (Wise and Rompre, 1989). However, unlike DA release activated by natural rewards, cocaine and amphetamine increase dopamine release in a more prolonged and unregulated manner (Nestler, 2005; 1997). Studies in primates indicate that the concentration of cocaine found in the brain is five to ten times higher than plasma values even after 2 hours post drug challenge (Benuck et al., 1988; Nayak et al., 1976). These drugs are highly lipophilic and freely cross the blood-brain barrier and bind to receptors with varying affinities found throughout the brain. The region of highest density and greatest affinity for drugs like cocaine are located in the cell bodies of VTA where they bind to dopamine transporters (DAT) used for the reuptake of DA back into the presynaptic neurons (Fowler et al., 1989; Sora et al., 1998; Rocha et al., 1998). Thus, cocaine inhibits the innate biofeedback mechanism which leads to significant elevation in NAc DA concentrations over baseline levels as measured by microdialysis (Hernandez and Hoebel, 1988; Pettit and Justice, 1989). Repeated exposures to these drugs contribute significantly to long-lasting adaptations in the brain's reward pathway leading to tolerance, dependence, craving, and repeated relapse (Kalivas and O'Brien, 2008). Further, it has been shown that there is a

positive correlation between the potencies of drugs of abuse as dopamine reuptake inhibitors and their ability to maintain behavioral sensitization (Volkow et al., 1998; Gately et al., 1997)

Other brain regions involved in the addiction process

Although, up to now, I have described the rewarding effects of drug exposure restricted to the VTA-NAc pathway, drug addiction likely involves changes in many brain structures (Kalivas and O'Brien 2008; Glowa et al., 1997; Kalivas and Duffy, 1998). Changes in the amygdala and hippocampus are important in mediating drug craving and relapse triggered by cues associated with past drug use (e.g., drug paraphernalia, locations associated with drug use) (Childress et al., 1999; O'Brien et al., 1990), while changes in frontal cortex may be particularly critical for the loss of control over drug use, a basic feature of addiction (Goldstein and Volkow, 2002; Volkow and Fowler 2000; Volkow et al., 1991)

Rewarding effects of solvents

Toluene abuse

Inhalant abuse in the United States trails only alcohol, marijuana, and nicotine abuse (Basu et al., 2004). Yet, relatively little is known about how organic solvents produce rewarding effects that lead to their abuse (Bowen et al., 1999; Balster, 1998). One way to increase knowledge in this area is to systematically compare the effects of abused solvents to those of other drugs of abuse that have been widely studied. Animal

models are particularly important in this regard due to health and safety concerns in a clinical environment. What's more, studies of solvents have been hampered by adequate methods to deliver controlled amounts of vapors. Therefore, I developed a novel inhalant chamber to study the neurobehavioral effects of toluene, the main psychoactive compound in abused solvents, to test the hypothesis that: (1) toluene alters reward / reinforcement learning behaviors in rodents, (2) the limbic system plays a role in toluene-induced reward / reinforcement behaviors, and lastly, (3) toluene induces synaptic DA release in the striatum of rodents.

Animal models of drug abuse

Over the past several decades, the behavioral abnormalities that are used to define addiction have been modeled successfully in animal models (Swerdlow et al, 2000). The availability of such models has made it possible to investigate the circuits and neurotransmitters involved in drug-seeking behavior. The three classic behavioral animal models, known to be regulated by the mesolimbic dopamine system, are self-administration, drug preference, and motor behavior [for review, see (Shippenberg and Koob, 2002)].

Self-administration

An extensive body of literature demonstrates that enhanced dopamine transmission is critical for the reinforcing effects of drugs of abuse [for review, see (Koob et al., 1998)]. A range of laboratory animals will strain for a range of drugs of abuse (e.g. cocaine, heroin, nicotine, alcohol) administered orally, intravenously, or by inhaling in a

drug self-administration model (Blokhina et al., 2004; Rocha et al., 1998; Wise and Bozarth, 1981; Roberts et al., 1977). Here, the animal performs some task, as pressing a lever, to trigger the administration of a drug often through an indwelling catheter. Dopamine receptor (D1 and D2) antagonists and agonists reliably modulate cocaine self-administration (Caine and Koob, 1994; Wise et al., 1990; Self et al., 2000; Platt et al., 2001). Further, depletion of NAc-DA via legions in the nerve terminals (Caine and Koob, 1994) or DA cell bodies in the VTA (Roberts and Koob, 1982) attenuates the self-administration of cocaine. These studies suggest that DA transmission in the NAc plays a crucial role in cocaine self-administration. However, not all drugs abused by humans can be modeled successfully in laboratory animals (e.g. hallucinogens) due to species-related or strain differences and other related factors [for review, see (Shippenberg and Koob, 2002)].

Conditioned place preference

Conditioned place preference (CPP) is a well established animal model to measure the reinforcing or aversive properties of abused drugs (Tzschentke, 2007; Shippenberg and Koob, 2002; Sora et al., 1998). In this procedure, a drug is paired with a particular environmental cue such as chamber color or floor texture; when tested later in the absence of the drug, the amount of time in the drug-paired environment determines whether the drug was reinforcing, neutral, or aversive. Interestingly, after a number of test sessions conducted in the absence of the drug, animal's no longer exhibit a place preference to the drug-paired environment; however, a priming dose (1/10th of the original dose) of the same drug or a stressful event can trigger the animal to reinstate their

preference for the drug-paired environment, as much as months after abstention (Shaham et al., 2000; See et al., 2003; Piazza and Le Moal, 1996). These behavioral studies reveal the complexity of drug use involving learning, conditioning, and memory which contribute significantly to addiction and repeated relapse (Adcock et al., 2006; Shaham and Hope, 2005; See et al., 2003). The conditions (location of drug abuse and associated people) in which the addict abused the drug are powerful cues in motivating use that becomes compulsive and leads to relapse in abstaining drug addicts. However, whether a lack of a conditioned response indicate a loss of reinforcing effects of the drug or an impairment of learning and memory processes to perform the conditioned task remains to be seen.

Psychostimulant locomotor activity

There is evidence that locomotor sensitization provides a useful animal model for drug reinforcement and addiction. Interestingly, animals that display high locomotor activity levels in a novel environment are more likely to produce drug-seeking behavior to psychostimulants, including cocaine, amphetamine, and nicotine (Suto et al., 2001; Piazza et al., 1989; Hooks et al., 1991b). Thus, locomotor sensitization provides a good model to study the inherent factors that drive an individual's risk for drug addiction (Hooks et al., 1991a). Furthermore, exposures to psychostimulants cause hyperactivity in a dose-dependent manner which can be attenuated by D2 receptor antagonist (Mattingly et al., 1994). In fact, studies in rodents indicate that exposure to reinforcing doses of cocaine (25 mg/kg) produce locomotor sensitization which highly correlate with increased extracellular NAc-DA release (Hooks et al., 1991b; Pettit and Justice, 1989;

Hernandez and Hoebel, 1988). This transient molecular and cellular response to psychostimulant administration models in animals the beginning stages of drug addiction, termed initiation.

Positron Emission Tomography

Imaging the addicted brain

A powerful tool to measure neurotransmission in living subjects is Positron Emission Tomography (PET), an imaging technique that uses short-lived positron-emitting radiotracers to monitor the distribution and pharmacokinetics of drugs (Fowler, et al. 1999; Wong et al., 1986; Phelps, 2002). This technique can be used to image and examine changes in brain function in the same subject from development to senescence. A wide variety of compounds have been radiolabeled to measure biochemical and physiological parameters. Most PET studies to evaluate addiction have focused on the dopamine synapse and brain metabolism. For example, endogenous dopamine has been assessed using [(11C)raclopride], a D2 receptor antagonist. (Gatley et al. 2005; Logan et al. 1991). The general principle is that drugs that increase extracellular dopamine release, decrease [(11C)raclopride] binding to postsynaptic D2 receptors and the opposite effect for drugs that decrease synaptic DA release. Changes in [(11C)raclopride] are based on the idea that endogenous DA competes for the D2 receptor binding site. The competition model has been reproduced in multiple studies to date (Tsukada et al., 1999; Ginovart et al., 2002; Mukherjee et al., 1997). Amphetamine is known to increase extracellular dopamine by inhibiting DA transporter protein (Sulzer et al., 1995). Studies have shown that manipulations that counter act amphetamine-induced DA release, like blocking the

DA transporter with GBR12909, attenuates amphetamine-induced decrease in [(11C)raclopride] binding (Villemagne et al., 1999; Ginovart et al., 2004). These studies show that changes in endogenous DA relates to changes in [(11C)raclopride] binding. However, alternative explanation to the competition model has been proposed. The strongest hypothesis is the internalization model (Skinbjerg et al., 2009; Laurelle M 2000) which suggests that DA receptors are internalized upon amphetamine challenge, thus, the decrease in [(11C)raclopride] is not due to competition but is due to a regulatory process of cellular activity to decrease further stimulation. Thus, the relationship between endogenous DA and [(11C)raclopride] binding might be more complex then originally hypothesized (Ginovart, 2005).

Alternatively, when dopamine is depleted with drugs that inhibit dopamine synthesis, like α-methly-para-tyrosine (AMPT) and reserpine, [(11C)raclopride] can be used to measure D2 dopamine receptor density (Ginovart et al., 1997; Laruelle et al., 1997).Under baseline conditions, this radiotracer takes advantage of the fact that D2 receptors are richly expressed in the striatum (STR), but poorly expressed in the cerebellum (CB); therefore, the STR to CB ratio (specific binding to the non-specific binding) which is also termed distribution volume ratio (DVR) can be compared between datasets (Logan et al., 1996; Patlak et al., 1983).

Different radiotracers can be used to assess the effect of drugs on brain function, including metabolism, neurotransmitter activity, enzyme activity, transporter or receptor occupancy. The most widely used radiotracer utilized to measure metabolic function is [18]FDG (^{18}fluro-D-glucose) (Reivich et al., 1979). This radiotracer allows the analysis of changes in brain metabolism in addicted subjects, longitudinally. For example, studies

in cocaine withdrawal subjects show reduced frontal metabolism measured 4 months after abstention (Volkow et al., 1993). The technique exploits the basic principle of glycolysis. Glucose transport proteins (GLUT) in the cell membrane transport glucose and [18]FDG into the cell. Glucose and [18]FDG are phosphorlyated by hexokinase as the first step in glycolysis. The enzymes that further metabolize glucose-6-phosphate cannot use [18]FDG-6-phosphate as a substrate, thus, [18]FDG-6-phosphate is trapped in the cell. This allows the analysis of the regions that are most sensitive to the effects of the drug. And, because the studies are done in awake human subjects, this allows the analysis of brain and behavioral relationships in addicted or non-addicted subjects. Today [18]FDG is the most widely used PET-tracer for basic research and clinical diagnosis.

Schizophrenia and substance abuse comorbidity

Schizophrenia

It was not until 1968 that the criteria of the operational diagnosis of schizophrenia were classified in the *Diagnostic and Statistical Manual of Mental Disorders* [DSM-II; American Psychiatry Association (APA, 1968)]. The major features of schizophrenia were classified into three symptoms – positive, negative, and cognitive. The positive symptoms of schizophrenia describe the psychotic episodes experienced with the disorder, like 'persecutory' delusions, hallucinations, and paranoia. The second subtype, the negative symptoms closely resemble depression which includes flat affect, social dysfunction, withdrawal, and lack of motivation. And third subtype is termed to describe dysfunctions of cognition, for example, thought disorder, memory loss, disorganized

speech or behavior. It is worth noting that the spectrum of symptoms of schizophrenia are simply dysfunctions of natural biological mechanism in which the brain filters, prioritizes, and processes the plethora of information from its internal and external environments. Therefore, schizophrenia research may represent the understanding of essential circuits that underlie all brain / behavioral processes. The etiology of schizophrenia is unknown; however, many theories have been proposed in the last few decades. Here, I will present the etiological research that focuses primarily on the likely causes and correlates of drug use in the schizophrenic population with the notion that no one single variable or set of variables can explain drug use by an individual.

Dopamine hypothesis

Dopamine was the first neurotransmitter system to be strongly implicated in schizophrenia. The DA theory of schizophrenia came from the observation that neuroleptics, which block DA transmission, alleviated psychotic, mostly positive, symptoms (Seeman et al, 1987; Snyder et al., 1973). Also, the clinical potencies of these drugs were found to be correlated highly with their affinity for DA receptors (Smith, Wolf, et al. 1988; Creese et al., 1976). Specifically, D2 receptors have a high affinity for antipsychotic drugs and are thought to be major sites of therapeutic action of these drugs. These studies indicate that schizophrenia may be mediated by hyperdopaminergic state (excess dopamine). However, why neuroleptic-induced therapeutic effects are not seen for several days or weeks did not support the DA surplus theory and that roles of other neurotransmitters must be at play (Snyder et al., 1974). However, more support for the DA theory came from studies using drugs that increase DA levels, like amphetamine.

These studies showed that DA release is elevated in drug-naïve schizophrenic patients compared to appropriate controls, and that DA levels directly correlated with induction of psychotic symptoms (Laruelle et al. 1995, 1997; Abi-Dargham et al., 1998). Moreover, a closer look at the postsynaptic receptors revealed higher expression of dopamine receptors in the striatal postmortem tissue of schizophrenic patients compared to the same region in normal subjects (Seeman, 1987). In living subjects, PET studies reported as high as threefold elevation of D2 receptors in the basal ganglia of schizophrenic patients compared to controls (Volkow, et al., 1991; Wong et al., 1986), while another group failed to reproduce these results. Nonetheless, it is highly probable that some aspects of schizophrenia symptoms are dopamine-mediated.

Genetic predisposition

Schizophrenia is a highly heritable disorder (Role and Talmage 2007; Harrison and Law, 2006; Harrison and Weinberger, 2005). In the last decade, the field of behavioral genetics and psychiatric genetics has converged on the importance of genes to explain psychological trait differences between humans. Evidence providing the roles of genes in mental disorders was derived mainly from twin studies showing a higher concordance rate in identical twins (41 - 65 %) compared with dizygotic twins (0 – 28 %) in schizophrenia (The Minnesota Study; Bouchard et al. 1990; Lykken et al. 1990), leading to heritability estimates as high as 85 % (Tsuang et al., 2001). However, differentiating genetic factors from environmental ones has made it difficult to interpreting these data. Thus, the question remains, whether schizophrenia represents one

disorder or is a set of disorders possibly involving gene-environment interactions remains a question and is an area of active research.

Multiple loci at different chromosomes have been identified as risk genes to schizophrenia [for review, see (Sklar, 2002)]. Linkage analyses of families with schizophrenia have revealed single-nucleotide polymorphisms (SNPs) on chromosome 8p, which codes for the neuregulin 1 (*NRG1*) gene (Stefasson et al., 2002, 2003; Blouin et al., 1998). The *NRG1* gene is involved in regulating neural signaling, by binding its family of tyrosine kinase transmembrane receptors ErbB that activates numerous intracellular pathways critical for development of the nervous system – cell proliferation, differentiation, and motility [for review, see (Mei and Xiong 2008)]. NRG1 isoforms have been divided into three families (type I, II, and III). Type III NRG1 isoform is unique in many ways. First, the expression of the type III is limited to neurons (Yang et al., 1998). Regions include the prefrontal cortex, hippocampus, cerebellum, and substantia nigra in both human and rodent brain (Kerber et al., 2003; Law et al., 2004). Second, the Type III NRG1 isoform can act both as a ligand for ErbBs while acting intracellularly as a transcription activator. More specifically, the Type III NRG1 isoform activates ErbB receptor on target cells and participates in juxtacrine signaling which is the classic "forward" signaling response. In addition, the unique C-terminal domain (NRG1-ICD) can participate in "back" signaling via a γ-secretase cleavage and subsequent nuclear translocation that alters gene expression of neurotransmitter systems, like the α7 nicotinic acetylcholine receptors (nAChRs) (Bao et al., 2003) and glutamatergic transmission (Fischbach, 2007) whose function is often misguided in

schizophrenia. These studies indicate that subtle changes in the Type III NRG1 signaling could alter key brain circuits involved in the etiology of schizophrenia.

Transgenic animal model for comorbidity

An important approach to clarify the functional roles of genes like *NRG1* has been through the phenotyping of genetically mutated mice (Duffy et al., 2008; Roy et al., 2007). Interestingly, targeted knockout of *Nrg1* in mice has been characterized to share endophenotypes (traits that have a clear genetic connection) with schizophrenic patients on multiple levels, from the molecular, physiological, and behavioral levels. In general, *Nrg1* mutant mice exhibit hyperlocomotor activity in an open field (Karl et al., 2007; O'Tuathaigh et al., 2007), have deficits in pre-pulse inhibition (PPI) which is an assay for sensorimotor gating (Chen et al., 2008), have reduced working memory (short term memory) (Chen et al., 2008), and altered social interactions (Karl et al., 2007). The phenotyping of Type III *Nrg1* mice, in particular, indicate that the homologous mutation results in embryonic lethality; however, the heterozygotes are viable and fertile (Wolpowitz et al., 2000) which has provided a unique opportunity to understand the effects of reducing NRG1 expression by half on CNS function in adult mice. Interestingly, PPI deficits observed in Type III *Nrg1* mice were ameliorated by nicotine, much like the reported effects of nicotine to alleviate symptoms of schizophrenia (Leonard et al., 2007). In addition, the molecular and anatomical endophenotypes of the Type III *Nrg1* mice – myelin defect, synaptic dysfunction, and ventricular enlargement – resemble pathological dysfunctions seen in schizophrenic patients (Chen et al., 2008; Hall et al., 2006).

However, no studies, to date, have examined neural areas associated with drug reward in the Type III Ng1 (+/-) mouse model of schizophrenia. Naturally, Type III Ng1 (+/-) mice gave me an opportunity to characterize the mesolimbic DA system in mice genetically predisposed to schizophrenia, to test the hypothesis that genetic perturbation of the *Nrg1* gene could affect increased sensitivity to DA agonists, and thus, vulnerability to drug addiction.

Experimental Objectives

As described above, perturbations in the limbic system due to drug abuse and/or genetic predisposition can lead to profound alterations in the reward circuit and addiction behavior. Studies presented in this thesis demonstrate that the rewarding effects of inhaled toluene are mediated, in part, by the dopamine system. In conjunction, toluene produced behavioral sensitization that is analogous to other well characterized CNS stimulants. Additionally, *in vivo* analyses of Type III *Nrg1* heterozygous mutant mice demonstrate that Type III NRG1 signaling affects dopaminergic function and sensitization to drug abuse.

In the first part of my thesis, I explore the reinforcing effects of inhaled toluene in an animal model of drug-seeking behavior. In Chapter 3, I demonstrate that inhaled toluene alters reward / reinforcement learning behaviors in a dose-dependent manner Next, in Chapter 4, I examine the metabolic signature underlying reinforcement learning behaviors in rodents to compare analogues processes that take place in human craving studies. In Chapter 5, I present data indicating that reinforcing doses of toluene produce marked decreases in metabolic function and aberrant brain pathology.

In the second part of my thesis (Chapter 6), I demonstrate that Type III *Nrg1* heterozygous mutant mice are more sensitized to the behavioral effects of cocaine. Further, drug-naïve Type III *Nrg1* heterozygous mutant mice show elevated D2 receptor density and increased dopamine metabolism indicating that Type III NRG1 signaling has an affect on basal dopaminergic function.

CHAPTER 2

Materials and Methods

Subjects: All animals use procedures were in strict accordance with the National Institute of Health guide for the care and use of all laboratory animals and were approved by the local animal care and use committee. For the first part of my thesis, studies utilized experimentally naïve, male, Sprague Dawley rats (Taconic Farms, Germantown, NY) that weigh 150–250 g and were given food and water *ad libitum*. Animals were housed in a vivarium with the temperature and humidity was kept relatively constant. All animals were housed in pairs and kept on a 12/12-light/dark cycle. Training and testing were conducted during the light cycle.

Further, Type III deficient *Nrg1* heterozygous mice ($Nrg1^{tm1.1Lwr}$) (Wolpowitz, 2000) and their wild type (C57/B6; Jackson Laboratory) littermates weighing between 25-29 g will be housed at Stony Brook University DLAR and transferred to Brookhaven National Laboratory as needed. Animals will be maintained on a 12 h light/dark cycle and provided food and water *ad libitum*. All animal procedures were conducted during the light cycle and were in strict accordance with the National Institutes of Health guide for the care and use of all laboratory animals and were approved by the local animal care and use committee.

Conditioned Place Preference Apparatus: The conditioning apparatus (ENV-013, MedAssociates, St. Albans, VT) consisted of three distinct compartments separated by two guillotine doors. The walls of the middle chamber were gray with a smooth floor,

while one conditioning compartment (21 × 21 × 27.5 cm, internal volume 12 l) had black walls with a smooth floor and the other one had white walls with a steel mesh floor. The lids of all three compartments were made of transparent plexiglass. The time spent in every compartment during test sessions was automatically recorded by infrared photocells positioned along the walls at the level of animal's head (six on both sides of the two conditioning compartments and three on both sides of the middle compartment) interfaced with MED-PC for Windows Version IV and Delphi TM 4 (SOF-735) (MedAssociates). The beams were arranged such that when an animal enters a chamber (defined as forepaws and the entire head in the chamber), the beam is broken and the timer begins recording. Once the animal leaves the chamber, the timer stops. In addition, the circuitry has an internal timer that shuts off all the beams after 15 min, which is the duration of the test session. The response of the computer to the break of a beam by an animal's body occurs within a tenth of a second. Accordingly, as an animal moves along from one beam to another the recording of the new position starts as the recording of the old position is ended. The cumulative data are presented as the number of seconds (tenths of seconds were eventually rounded for the clarity) spent in each chamber.

The apparatus was modified to allow for dynamic vapor exposure. To render the exposure chambers airtight, the lids were equipped with rubber gaskets and clasp locks. An opening on the top of the side of both black and white compartments was used to introduce toluene vapors under positive pressure, and the atmosphere was exhausted through an opening at the bottom of both chambers, insuring that atmospheric pressure within the chamber was not altered by the introduction of the toluene vapor. The flow

of vapors was initiated at least an hour prior to the beginning of the exposure to allow for chamber concentrations to equilibrate, as confirmed by air sampling. Animals were quickly introduced into the chambers by opening the lid, which was immediately closed and the chamber resealed while toluene vapors continued to be introduced for the remaining of conditioning session.

Measurement of Toluene Concentrations: Toluene (99%) was purchased from Sigma-Aldrich (Mil- waukee, WI). Mixtures of toluene vapors and air were metered using two mass flow controllers (Dyna-Blender by Matheson Tri-Gas, Mongomerville, PA) with a total flow of gas mixture set at 2 l/min. Pure laboratory air was metered through one flow controller while the second one metered air that was bubbled through a 500 ml round bottom flask filled with toluene set either in a common flask holder (room temperature) or in an insulated ice bath (used only for creating 810 ppm vapor levels). All connections were made using 0.25 inch outside diameter stainless steel tubing. Blending the two gas streams created variable concentrations of toluene vapors that were continuously monitored by partitioning a small portion of the gas stream through a 1/32 inch outside diameter stainless steel tube interfaced with a flame ionization detector (SRI Instruments, Torrance, CA). The signal output from this detector was analyzed using PeakSimple software (SRI Instruments).

The on-line flame ionization detector response to various levels of toluene vapors was calibrated using a capillary gas chromatograph (Hewlett Packard 5690A) equipped with a 60 × 0.25 mm i.d. SE-30 capillary column maintained initially at 50 °C, with the temperature programmed to reach 150 °C at 3 °C/min rate at the time of

sample injection. Helium was used as a carrier gas.

For calibration purposes, toluene vapor samples were collected from the tubing outlet connected to the chambers by bubbling the gas stream through a vial with 10 ml of hexane for 2 min. To insure that trapping of toluene was quantitative, parallel measurements were obtained using 100 ml vials of hexane with collection periods increased to 10 min. No differences were noted between the two sampling times, suggesting that all toluene was trapped in the 2 min sample. This also then served as a systematic replication of the calibration curves.

Toluene peaks were analyzed and integrated using a Vision 4 Chromatography Acquisition station. The integrated peaks (in peak-area-units) were subjected to a linear regression analysis and the resulting equation was used to convert peak-area-units to nM and subsequently parts per million (ppm) of toluene.

In order to independently verify toluene levels created in the exposure chambers and insure that the levels were uniform, nine small holes (three for each level: top of the chamber, level of animal's head, and 2 cm above the floor) were drilled in the walls of both boxes. Air samples were drawn with a gas-tight syringe and were immediately dispensed into vials containing hexanes to trap the toluene vapors. The amount of toluene was then determined from the calibration curve.

Mixing the gas streams of toluene and pure air in the proportion of 1.0 l/min of pure air and 1.0 l/min of toluene kept at room temperature yielded an average toluene concentration inside the chamber of 4950 ppm. A mixture of 1.5 l/min of pure air and 0.5 l/min of toluene produced an average toluene concentration of 1895 ppm. We obtained an average toluene vapor concentration of 810 ppm with a mixture of 1.95

l/min pure air and 0.05 l/min of toluene kept at 0 °C to retard evaporation.

It should be noted here that the actual concentrations of toluene vapors obtained inside the chamber are the function of the step-wise changes in the ratio of two gas flows dictated by the design of the flow meters used in our experiments.

Measurement of Acetone Concentrations: An air stream saturated with acetone vapor was generated by bubbling air through a flask containing liquid acetone maintained at 0 °C. This air/acetone saturated stream was diluted with compressed air in predefined ratios set by computer-controlled flow regulators (Dyna-Blender, Matheson, PA). For this calibration acetone vapors were introduced at 2 l/min for at least 1 h so that the chamber volume (12 l) was exchanged a minimum of 10 times. In order to independently verify that acetone levels created in the exposure chambers and ensure that the levels were uniform, nine small holes (three for each level: top of the chamber, level of animal's head, and 2 cm above the floor) were drilled in the walls of both boxes. Air samples were drawn with a gas-tight syringe and were immediately dispensed into vials containing water to trap the acetone vapors.

Acetone vapor concentrations were measured with a gas chromatograph using a 1/8 in Porapak T column. Acetone peaks were analyzed and integrated using a Vision 4 Chromatography Acquisition station. The integrated peaks (in peak-area-units; PAU) were subjected to a linear regression analysis and the resulting equation was used to convert PAU to nM and subsequently parts per million (ppm) of acetone. This resultant standard curve of acetone concentrations was used to derive and maintain the chamber concentration as a function of the combined air and acetone bubbler flow rates. For these studies, we obtained an average acetone vapor concentration of 5000 ppm with a mixture

of 1.80 l/min pure air and 0.20 l/min of acetone. Mixing the gas streams of acetone and air in the proportion of 1.75 l/min of air to 0.25 l/min of acetone produced an average acetone concentration of 10,000 ppm. A mixture of 1.63 l/min of air and 0.37 l/min of acetone produced an average chamber concentration of acetone of 20,000 ppm. Acetone was maintained at 0 °C in an ice bath at all concentrations to retard evaporation.

Conditioned Place Preference Procedure: Pre-conditioning phase: during the first two preconditioning days, the animals were transported from the Brookhaven Laboratory Animal Facility to the Chemistry Department building in order to get acclimated to the transportation procedure and to the test room. The animals were transported daily at 12 pm in their home cages, two animals per cage. On the third pre-conditioning day, the animals were allowed to freely explore all three compartments of the conditioned place preference chamber for 15 min and the time spent in each compartment was electronically recorded to assess the unconditioned preference for either one of the distinctive side compartments. On average, the animals spent approximately an equal amount of time (mean ± S.E.M. seconds) in both white and black chambers (black: 286 ± 22 s; white: 291 ± 26 s) in this pre-conditioning session. Thus, the apparatus was truly unbiased in terms of chamber preferences in untreated rats.

Conditioning phase: during the following conditioning phase (12 days, six pairings for each treatment, consecutive days, including weekends), rats were assigned to receive toluene (810, 1895, 3000 or 4950 ppm) or air in one of the two compartments in a counterbalanced fashion, with half of the animals receiving toluene in the white compartment and air in the black one and the other half receiving toluene and air in an

opposite fashion. At 1895 and 3000 ppm conditioning was continued for an additional 6 sessions over 4 weeks. During each training session, animals were confined for 30 min to one side of the apparatus with the doors closed. Cage mates were exposed to the training drug in pairs, so that on any given conditioning day, both sides of the apparatus were filled with either air or toluene. The control group was exposed to air in both compartments. Fecal pellet output rate in each compartment was used as an indicator of stress and recorded at the end of each session. The last day before the test day was always assigned to be an air exposure conditioning session.

Testing phase: on the first test day (the next day after the last training session), animals were placed in the middle compartment of the conditioned place preference chamber for 5 min with the guillotine doors closed for an initial acclimation. Subsequently, the doors were raised and the animals were allowed free access to all three compartments for 15 min. The time spent in each compartment (white/gray/black) was electronically recorded. A locomotor activity score was obtained by recording the total number of chamber crossing. Due to technical problems, these scores were obtained only in the animals in the 1895 and 810 ppm toluene groups

On the second test day, the animals were brought back and again allowed free access to all three chambers. However, this time, the apparatus was filled with the training concentration of toluene. The control group of toluene-naïve animals was pre-conditioned and tested under an identical protocol, except that toluene was omitted on conditioning sessions.

<u>CPP Statistical Analysis</u>: Conditioned place preference test outcomes for each concentration were determined by the time spent in the chamber paired with toluene

compared to the chamber paired with air. A positive conditioned place preference for a particular dose of toluene was indicated by a significantly ($p < 0.05$, paired two-tail t-test) greater mean time spent on the toluene-paired side than on the air-paired side. The preference score was defined as the difference between the mean time spent in the drug-paired chamber minus the mean time spent in the air-paired chamber. For the locomotion data, the number of chamber transitions on the first test day was compared for the same group of animals with the number of crossings on the second test day when toluene was administered. For determination of the effect of the number of conditioning trials on the place preference, preference scores (time spent in drug-paired minus time spent in vehicle-paired) were analyzed with a two-factor analysis of variance (ANOVA) with a one factor being the number of conditioning trials and the second factor the concentration of toluene.

Animal PET: Imaging was performed using an R4 tomograph (CTI, USA), which has a transaxial resolution of 2.0 mm full width at half maximum (FWHM). Animals were anesthetized with ketamine and xylazine (10:1) and catheterized for lateral tail vein injection of ~798 uCi [(11)C]raclopride corresponding to an injected raclopride mass of ~1.5 nmol/kg. Each animal was positioned prone on the microPET bed with the brain in the center field of view (CFOV). Fully 3-D listmode data was collected for 60 mins starting at time of injection and histogrammed into 24 time frames (6×10 s, 3×20 s, 8×60 s, 4×300 s and 3×600 s). The resulting sinograms were reconstructed using Expectation-Maximization (EM) algorithm for maximum likelihood (ML) after randoms

subtraction. Scattered events were accounted for using a two tail-fit algorithm (Alexoff et al., 2003).

For measurements of glucose activity, animals were food deprived and brought to the microPET facility approximately 3 hours prior to scanning. Animals were injected intraperitoneally (i.p.) with 600–1000 μCi (18)FDG 50 min prior to anesthesia. The 50 min time period was chosen to be sufficient for equilibrium between (18)FDG in plasma and brain. After the uptake period, animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Each animal was positioned prone on the bed with the brain in the center FOV. Fully 3-D listmode data was collected in a single time frame of 600 s. One blood sample was taken at the end of the scan to measure whole blood glucose levels.

Further, for simultaneous brain and behavior measurements, Sprague–Dawley rats received a baseline PET (18)FDG scan. Rats were separated into two groups: toluene group and saline group. Rats in the toluene group were alternately administered toluene vapors (3000 ppm, 12 pairings) or saline in distinct environments using a behavioral model of cue-induced reward (Conditioned Place Preference), whereas rats in the saline group were administered strictly with saline in the two distinct environments. Rats received a second (18)FDG scan during the place preference test. On the (18)FDG scan day (24 h after the final pairing session), rats were placed in the middle acclimation compartment of the CPP apparatus, injected with (18)FDG, and given access to all compartments during the 45 min uptake period. Time spent in each environment was electronically recorded and rated with a computerized automated system. Rats were then anesthetized and scanned for 10 min. (18)FDG data were normalized to stereotaxic space

and proportionally scaled to the global mean. Voxel-wise analysis was implemented in SPM2 and verified by Region of Interest (ROI) regression analysis performed using PMOD, where the main factor of interest was preference score (time in cocaine-paired compartment minus time in vehicle-paired compartment).

Animal PET Analysis: Images were reconstructed by a 3D maximum a posteriori (MAP) algorithm with 18 iterations and regularization parameter of 0.005, as previously described (Lee et al., 2006). A mean (18)FDG image was created in SPM2 (Statistical Parametric Mapping). Data from each animal was then coregistered to the mean (18)FDG image using a mutual information (MI) algorithm implemented with the Pixelwise Modeling (PMOD; Zurich, Switzerland) software package. The MLEM image pixel size was 0.4 mm × 0.4 mm × 1.21 mm (*x*, *y*, *z*). A ROI template was developed in PMOD based on a digital atlas of the rat brain (Paxinos and Watson, 1997) as previously described (Lee et al, 2006). Briefly, the ROI template was developed in PMOD based on a digital atlas of the mouse brain (Franklin, P. 1996) and included 10 regions; the frontal/prelimbic/cingulate cortex (FrA-PrL-Cg; with the center at ±0.9 mm lateral to bregma, *x*-axis; +1.6 mm anterior to bregma, *y*-axis; −3.1 mm below bregma, *z*-axis), primary motor cortex (M1; ±3.4, +1.3, and −3.1), sensory cortex (S1; ±4.3, −0.9, −4.6), auditory/temporal cortex (temporal, AuTeA; ±5.5, −5.3, −4.6), cerebellum (CB; ±1.9, −12.5, −6.0), caudate/putamen (CPu; ±2.2, +0.6, −5.6), thalamus (Thal; ±1.8, −3.2, −6.4), hippocampus (Hippo; ±2.4; −4.2, −4.0), midbrain (±0.2, −6.2, −6.3) and pons (±0.3, −8.5, −7.9). The mean volume of all ROIs was 0.86 cm^3 and the shapes were elliptical or round. Data were normalized for whole brain uptake and compared between datasets

using $P < 0.05$ significance threshold. Across subject variability was determined by Coefficient of Variable (CV = standard variation/mean). Statistical analysis included a two-way repeated measures analysis of variance (ANOVA), with ROI as one factor and age as the repeated measures factor. Statistically significant results were followed up using Student Neuman-Keuls post hoc tests. Lastly, for qualitative results, a mean image was created in Statistical Parametric Mapping 2 (SPM) software.

In addition, for the [(11)C]raclopride studies, the binding potential (Bmax/Kd) was computed using the graphical analysis method without blood sampling (Logan et al., 1996). The BP, which can be derived from the distribution volume ratio (DVR) using the equation BP = DVR-1, was used for comparing data sets. This metric is the ratio of the receptor (target protein) concentration to the dissociation constant, and has been shown to be a reproducible measure of dopaminergic function *in vivo* (Gatley et al. 1997).

MicroMRI: MR experiments were performed on a 9.4 T/21 cm horizontal magnet (Magnex, UK) interfaced to an AVANCE console (Bruker, Billerica, MA), and an 11.6 cm diameter BGA gradient insert (400 mTm^{-1}; actively shielded, 170 µs risetime; Magnex, UK). A 1.2 cm diameter surface coil was positioned above the animal and T2-weighted MR data sets were generated with a 3D gradient-echo pulse sequence [repetition time (TR) = 200 ms; echo time (TE) = 80 ms]. For these scans, the spatial resolution was 100 µm × 100 µm × 1.5 mm. For T2-weighted data sets, a 3D fast Fourier transform was applied without any apodization, filtering, or zero filling. In all cases, 3D data sets were rendered into 16-bit grayscale images for registration, segmentation, and other processing manipulations.

For MT images, a rapid acquisition relaxation enhancement (RARE) sequence was used based on parameters determined previously to obtain the best contrast between white and grey matter. The initial parameters used an offset frequency of 3000 Hz, amplitude pulse of 3 μT, TR = 3000 ms and TE = 15 ms. A reference sequence was obtained using the same parameters but without applying a saturation pulse. The MTR was calculated as: MTR = $(1-M_s/M_o) \times 100$, where M_s is the magnitude of signal under MT saturation and M_o is the magnitude of signal without MT saturation. To account for variation in the initial white matter volume, change in MTR over time was expressed as a percentage of that obtained from the original, pre-toluene scan.

Histology: After the second MRI imaging session (post-toluene exposure), animals were sacrificed with isoflurane (Sigma Aldrich). Brains were removed and postfixed in 4 % paraformaldehyde over night. Weil stain for myelin was applied to paraffin sections (Neuroscience Associates).

Immunoblotting: Strital tissue was lysed in RIPA buffer (Thermo Fisher Scientific) supplemented with 10 mM DTT, protease inhibitors (Sigma-Aldrich), and phosphatase inhibitors (Thermo Fisher Scientific). Lysates were be separated on 12 % SDS-PAGE gels and transferred to nitrocellulose filters. Filters were be blocked in 5 % milk in 0.1 % Tween-20 TBS solution at room temperature for 2 h before overnight incubation in primary antibody solutions in 5 % milk in Tween-20 TBS solution at 4 °C (anti-D2DR [1:500; Santa Cruz Biotech, Inc.] and anti-GAPDH [1:15,000]).

In Vivo Microdialysis: The mice were anesthetized with a ketamine/xylazine (10:1) cocktail; and placed in a standard stereotaxic frame using the standard adaptor for a mouse (Kopf). The scalp was incised (5-8 mm), the skull cleaned and two drill holes were made using a fine drill (0.7 mm diameter) for the fixing of the screws. Two microscrews were placed into the skull. Another hole (2 mm diameter) was drilled directly above the NAc, +1.5 mm anterior and ±0.8 mm lateral to bregma according to the mouse atlas (Franklin, P. 1996). A guide cannula 15 mm in length was lowered 4.8 mm below skull surface and secured with dental acrylic resin. After the resin was sufficiently dried, a CMA membrane probe (1.0 mm) was positioned within the guide cannulae and perfused with artificial cerebrospinal fluid (148 mM NaCl/2.7 mM KCl/1.2 mM $CaCl_2$/0.85 mM $MgCl_2$, pH 7.4) at a constant flow rate of 1.0 µl/min. Samples were collected every 20 min and analyzed for dopamine (DA), homovanillic acid (HVA), and 3,4-dihydroxyphenylacetic acid (DOPAC) by high-performance liquid chromatography (HPLC) coupled to an electrochemical detection.

Neurotransmitter Analysis: The HPLC system (PM 92-E pump, BAS, West Lafayette, IN) via an on-line autoinjector (Pollen-8, BAS, West Lafayette, IN) consisted of a microbore column (150 × 1 mm, 5-m C18 column; BAS), a BAS LC-4C electrochemical transducer with a dual glassy carbon electrode set at 650 mV relative to an Ag/AgCl reference electrode, a computer that analyzes data on-line by using a commercial software package (BAS). The mobile phase (flow rate 1.0 ml/min) consisted of the mobile phase consists of 14.5 mM NaH_2PO_4, ; 30 mM Sodium citrate; 10 mM diethylamine HCl; 2.2 mM 1-octanesulfonic acid; .027 mM EDTA; 7.2 % acetonitrile (v/v), 1 % tetrahydrofuran

(v/v), pH 3.4., for catecholamine analysis. The overall detection limit of is usually somewhere between 8-12 fmol. Dopamine peak identity was confirmed and quantified in relation to standard peaks of known concentrations (x-y ng/ul) run through the HPLC system on the same day as the animal data were collected; uncorrected peak values are measured automatically by ChromGraph software (BAS, West Lafayette, IN, USA) as peak area/heights and were converted to concentrations (fmol/μl) using the standards.

CHAPTER 3

An Animal Model for the Pharmacological and Behavioral Effects of Solvent Abuse

Abstract

Toluene is a ubiquitous ingredient in household products (e.g. glue, air fresheners, adhesives, nail polish, and paint) that has putative abuse liability. However, there is little empirical evidence about how the chemical and biological properties shared by other volatile solvents relate to their abuse potential. Here I explore if conditioned place preference can be used to study the rewarding effects of inhalants in rats. Sprague-Dawley rats were confined to a distinct compartment of a three compartment chamber while exposed to toluene or air and later tested for preference for the toluene-paired environment. Identical studies were performed with inhaled acetone. Rats that received air in both sides (control group) did not show any preference for either side with approximately equal time spent in each compartment on the test day. Further, rats spent equal time in the acetone- vs. air-paired compartment. However, the 1895 and 3000 ppm toluene test groups demonstrated a significant preference for the toluene-related environment. This procedure should be useful for further studies of abuse-related similarities and differences among volatile solvents.

Introduction

Solvent abuse is a significant worldwide problem associated with serious health and social cost (Anderson and Loomis 2003; Brouette and Anton, 2001; Howard and Jenson, 1999). The neurological and psychiatric consequences of inhalant abuse include paranoid psychosis (Byrne, Kirby et al. 1991), parkinsonism (Uitti et al., 1994), cognitive impairment (Evans and Raistrick 1987; Chadwick et al., 1989; Chadwick and Anderson, 1989) and cerebellar dysfunctions (Kamran and Bakshi, 1998; King, 1982). In addition, inhalant abuse is associated with a substantially increased likelihood that adolescents will develop other substance abuse disorders (Cannon, Cadenhead et al. 2008; Dinwiddie 1994; Howard and Jenson 1999; Dinwiddie et al., 1991a,b; Johnson et al., 1995; Schutz et al., 1994). Though inhalants produce many psychological and behavioral effects similar to those of other abused drugs and meet the *Diagnostic and Statistical Manual of Mental Disorders* [DSM-IV; American Psychiatry Association (APA, 1994)] diagnostic criteria for drug dependence (Howard et al., 2001; Kono et al., 2001; Miyata et al., 2001), the development of animal models for their abuse-related effects lags far behind progress with other classes of drugs of abuse (Balster 1998).

Abused solvents are almost always inhaled by substance abusers, and their very rapid distribution into the brain (Gerasimov, Ferrieri et al. 2005) may result in immediate reinforcing effects which contributes to their abuse liability and risk of dependency. Therefore, animal models, which utilize the inhalation route, are important for studying the abuse-related reinforcing and other central nervous system effects of these agents. Undoubtedly, the technical difficulties of producing controlled exposures to vapors have been limiting the development of these animal models.

Place preference conditioning is a valuable, firmly established, and widely used tool in behavioral pharmacology and addiction research (for review, see Tzschentke 2007; Bardo and Bevins, 2000; Tzschentke, 1998). I have chosen to utilize the conditioned place preference paradigm to measure reward-related behaviors in rats exposed to toluene or acetone, the main psychotropic components found in many abused solvents (Balster, 2003). To pursue this goal, my colleagues and I developed a novel, completely automated, dynamic vapor exposure conditioned place preference apparatus to investigate behavioral effects of inhalants.

The rewarding properties of toluene (810, 1895, 2995, and 4950 ppm) were examined in rats using a conditioned place preference paradigm with a three-compartment chamber. The choice of this dose range was based on the report by Bowen et al. (1996) showing that 600 - 1000 ppm is the minimal effective concentration for producing toluene's discriminative stimulus effects in mice. Accordingly, 10,000 – 12,000 ppm produces the symptoms of ataxia, slurred speech and hallucinations in toluene abusers (Garriott et al., 1981).

During conditioning, treatment animals were exposed to toluene or acetone in distinctive compartments, while control animals received air in the same apparatus. During test sessions, animals were initially placed in a middle compartment and allowed access to all three chambers. The time spent in the toluene-paired compartment was used to measure the place preference score (the difference between time spent in drug-paired vs. vehicle-paired compartments). Because the expression of a conditioned place preference can sometimes be greater when animals are tested when under the influence of the training drug (Bespalov et al., 1999), these studies compared test sessions

conducted with and without toluene exposure.

Lastly, locomotor activity was recorded by infrared photocells that lined the walls of the apparatus at the level of the animal's head to better understand locomotion as a function of treatment (toluene or acetone), vapor concentration, and the number of pairing sessions.

Experimental Design

Subjects: All animals use procedures were in strict accordance with the National Institute of Health guide for the care and use of all laboratory animals and were approved by the local animal care and use committee. These studies utilized experimentally naïve, male, Sprague Dawley rats (Taconic Farms, Germantown, NY) that weigh 150–250 g and were given food and water *ad libitum*. Animals were housed in a vivarium with the temperature and humidity was kept relatively constant. All animals were housed in pairs and kept on a 12/12-light/dark cycle. Training and testing were conducted during the light cycle.

Conditioned Place Preference Procedure: Pre-conditioning phase: during the first two preconditioning days, the animals were transported from the Brookhaven Laboratory Animal Facility to the Chemistry Department building in order to get acclimated to the transportation procedure and to the test room. The animals were transported daily at 12 pm in their home cages, two animals per cage. On the third pre-conditioning day, the animals were allowed to freely explore all three compartments of the conditioned place preference chamber for 15 min and the time spent in each compartment was electronically recorded to assess the unconditioned preference for either one of the distinctive side compartments. On average, the animals spent approximately an equal amount of time (mean ± S.E.M. seconds) in both white and black chambers (black: 286 ± 22 s; white: 291 ± 26 s) in this pre-conditioning session. Thus, the apparatus was truly unbiased in terms of chamber preferences in untreated rats.

Conditioning phase: during the following conditioning phase (12 days, six pairings for each treatment, consecutive days, including weekends), rats were assigned

to receive toluene (810, 1895, 3000 or 4950 ppm) or air in one of the two compartments in a counterbalanced fashion, with half of the animals receiving toluene in the white compartment and air in the black one and the other half receiving toluene and air in an opposite fashion. At 1895 and 3000 ppm conditioning was continued for an additional 6 sessions over 4 weeks. During each training session, animals were confined for 30 min to one side of the apparatus with the doors closed. Cage mates were exposed to the training drug in pairs, so that on any given conditioning day, both sides of the apparatus were filled with either air or toluene. The control group was exposed to air in both compartments. Fecal pellet output rate in each compartment was used as an indicator of stress and recorded at the end of each session. The last day before the test day was always assigned to be an air exposure conditioning session.

Testing phase: on the first test day (the next day after the last training session), animals were placed in the middle compartment of the conditioned place preference chamber for 5 min with the guillotine doors closed for an initial acclimation. Subsequently, the doors were raised and the animals were allowed free access to all three compartments for 15 min. The time spent in each compartment (white/gray/black) was electronically recorded. A locomotor activity score was obtained by recording the total number of chamber crossing. Due to technical problems, these scores were obtained only in the animals in the 1895 and 810 ppm toluene groups

On the second test day, the animals were brought back and again allowed free access to all three chambers. However, this time, the apparatus was filled with the training concentration of toluene. The control group of toluene-naïve animals was pre-conditioned and tested under an identical protocol, except that toluene was omitted on

conditioning sessions.

CPP Statistical Analysis: Conditioned place preference test outcomes for each concentration were determined by the time spent in the chamber paired with toluene compared to the chamber paired with air. A positive conditioned place preference for a particular dose of toluene was indicated by a significantly ($p < 0.05$, paired two-tail t-test) greater mean time spent on the toluene-paired side than on the air-paired side. The preference score was defined as the difference between the mean time spent in the drug-paired chamber minus the mean time spent in the air-paired chamber. For the locomotion data, the number of chamber transitions on the first test day was compared for the same group of animals with the number of crossings on the second test day when toluene was administered. For determination of the effect of the number of conditioning trials on the place preference, preference scores (time spent in drug-paired minus time spent in vehicle-paired) were analyzed with a two-factor analysis of variance (ANOVA) with a one factor being the number of conditioning trials and the second factor the concentration of toluene.

Results

Toluene place preference

Rats that received air in both sides (control group) did not show any preference ($p = 0.8$) for either the black or white side with approximately equal time spent in each compartment on the test day (mean ± S.E.M. of 241 ± 33 s and 234 ± 34 s, respectively). These results in the air control group are very similar to the results for all the animals when their preference was measured in the pre-conditioning phase (black: 286 ± 22 s; white: 291 ± 26 s, $p = 0.7$).

Animals conditioned with 810 ppm of toluene did not show a significant preference ($p = 0.7$) for any compartment (274 ± 24 s vs. 261 ± 26 s spent on toluene and air-paired side, respectively) (Fig. 3.1A, left panel). Exposure to this concentration of toluene on the second test day did not alter this outcome (291 ± 31 s vs. 302 ± 39 s) (Fig. 3.1A, right panel).

On the other hand, animals conditioned with 1895 ppm for 6 pairing sessions spent significantly ($p < 0.05$) more time on the toluene-paired side than on the air-paired side (299 ± 26 s vs. 201 ± 22 s, Fig. 3.1B, left panel). Exposure to this concentration of toluene on the second test day led to the loss of preference for the toluene-paired side in this group of animals. In fact, results showed that a trend toward increased time spent on the air-paired side that did not reach significance ($p = 0.2$) (215 ± 66 s vs. 406 ± 94 s for the toluene and air side, respectively) (Fig. 3.1B, right panel).

Extending the number of toluene pairings to 12 significantly enhanced the time

spent in the toluene-paired chamber over the air-paired chamber compared to that obtained following six pairings (Fig. 3.2) ($F(1,60) = 11.2$, $p<0.01$ for effect of pairings on place preference using a two-factor ANOVA, with one factor representing the number of conditioning pairings and one factor the toluene concentration). However, no preference was observed at the highest concentration of 4950 ppm.

Toluene effects on locomotor activity

There was no difference between chamber crossings in test sessions conducted with and without toluene in either the toluene-conditioned animals ($p = 0.14$) or toluene-naïve animals receiving toluene for the first time ($p = 0.06$) (Fig. 3.3). In the group of animals trained with 1895 ppm of toluene, locomotor activity was significantly ($p < 0.01$) higher when animals were exposed to toluene (Fig. 3.3B, left panel), as compared to their score obtained in toluene-naïve animals during test session (Fig. 3.3B, right panel). There were significant differences between test sessions conducted with 810 ppm toluene exposure and test sessions with 1895 ppm exposures. The number of chamber crossings was lower in the 1895 ppm exposures than with the 810 ppm exposures in both trained and naïve animals ($p = 0.002$ for both groups) (compare upper and lower panels of Fig. 2.3).

A stressful response (represented by fecal pellet output rate) in the animals exposed to 1895 ppm was observed on the first day of toluene conditioning which showed adaptation by the second pairing session (Fig. 3.4). Animals conditioned with 810 ppm of toluene did not display any changes in pellet output throughout the pairing

sessions compared with the air-trained control animals.

Acetone place preference

The combined pre-test data indicate that there was not a baseline chamber bias (mean ± S.E.M; black: 281 ± 22 s; white: 271 ± 26 s, $p = 0.4$). More detailed analysis indicated that animals which received 5000 ppm spent 310 ± 35 s on the black and 323 ± 26 s on the white sides ($p = 0.8$; Fig 3.6). Animals in the 10,000 ppm group that received 6 pairings spent 217 ± 19 s and 176 ± 32 s in the black and white chambers, respectively ($p = 0.3$). This same group of animals received an additional 6 pairings. Additionally at the 10,000 ppm level, animals paired with 3 pairing sessions spent 202 ± 25 s and 204 ± 18 s in the black and white chambers, respectively ($p = 1.0$). Further, animals in the 20,000 ppm group spent 315 ± 13 s in the black and 316 ± 19 s in the white compartments, $p = 1.0$. The pre-test data were similar to control animals that received air in both the black and white compartments on the test day (241 ± 33 s and 234 ± 34 s, respectively).

Acetone effects on locomotor activity

The locomotor activity for each acetone vapor concentration is depicted in Fig. 3.5 (A, B, C). A three-way ANOVA yield a significant effect of treatment ($F[1,287] = 73$, $p < 0.001$), exposure session ($F[5,287] = 23$, $p < 0.001$) and concentration ($F[2,287] = 72$, $p < 0.001$). Additionally, there was a significant interaction between exposure session and concentration [$F(10,287) = 2$, $p < 0.05$].

Discussion

Aromatic hydrocarbons are a major chemical constituents in household and industrial solvents contributing to the morbidity associated with inhalant use and abuse (Balster 1998; Bruckner and Peterson 1977). Since many different classes of chemicals are used on a routine basis as organic solvents, including aliphatic hydrocarbons, aromatic hydrocarbons, ketones, ethanol, and tetrachloroethylene (Evans and Balster 1993), it is critical to better understand how the physical and biological properties shared by organic solvents relate to their potential for abuse. An important goal of this study was to develop an animal model to quantify the abuse-related behavioral effects of inhaled toluene and acetone, the main components in abused solvents.

Three key results came from these studies: 1) the CPP test was successfully adapted to study inhalants, 2) rats exhibit a CPP to inhaled toluene in a dose-dependent manner, and 3) rats do not exhibit a CPP to inhaled acetone. Rats will show a CPP for a variety of drugs that are abused by humans (Tzschentke, 1998; Bardo and Bevins, 2000). Cocaine, amphetamines, and opiates produce especially robust place preferences (Carr and White, 1986; Nomikos and Spyraki, 1988). This represents the first reported study in rats using pure toluene administered during conditioning sessions by inhalation and extends the results from the previous two reports of toluene-induced conditioned place preference in mice (Funada et al., 2002) and in rats (Yavich et al., 1994) using mixed volatile solvents.

Conditioning sessions performed with 1895 and 3000 ppm toluene produced a significant conditioned place preference. The degree of preference observed here

is similar to the mean preference score of 180, 140, and 150 s previously reported for the group of mice exposed to 700, 2500, and 3200 ppm of toluene, respectively (Funada et al., 2002). This range of effective concentrations (1895-4950 ppm) corresponds to other studies of the behavioral effects of toluene in laboratory animals. These include changes in locomotor activity in mice (Bowen and Balster, 1998), cross-sensitization to the locomotor-stimulating effects of cocaine in rats (Beyer et al., 2001), direct effects on schedule-controlled responding in mice (Moser and Balster, 1985; Bowen and Balster, 1998), discriminative stimulus effects in mice and rats (Rees et al., 1987; Yavich et al., 1994), self-administration in monkeys (Weiss et al., 1979) and mice (Blokhina et al., 2001) and in decreased intracranial self-stimulation current intensity threshold in rats (Bespalov et al., 2003). Accordingly, similar concentrations of inhaled toluene produces regionally specific changes in extracellular brain dopamine levels in rats (Riegel et al., 2007; Gerasimov et al. 2002).

In the present study, the lower dose of 810 ppm toluene failed to produce a conditioned place preference. It is likely that this concentration is below the threshold for producing effects in the brain necessary for developing a basis for drug-seeking behavior. However, this finding should be considered in the context of species differences. That is, numerous behavioral studies in mice show that 600-1000 ppm of toluene is still effective in producing discriminative stimulus effects (Bowen et al., 1999), effects on locomotor activity and schedule-controlled behavior (Bowen and Balster, 1998), and in anxiety paradigms (Lopez-Rubaclava et al., 2000). Finally, Funada et al. (2002) observed a significant conditioned place preference in mice exposed to 700 ppm. Species differences between mice and rats have been reported before, for example, another

abused ‘‘solvent’’ ethanol produces conditioned place aversion in rats and a conditioned place preference in mice (Cunningham et al., 1993).

Higher toluene concentrations, in the range of 4950 ppm and above, produce anesthesia-like effects and strong sedation (Bowen and Balster, 1998; Beyer et al., 2001; D.E. Lee and A.N. Gifford, unpublished observations) and may be toward the high end of the well-established inverted u-shaped dose response curve.

I found that the toluene conditioned place preference was only evident on test sessions when toluene was not administered, and that it disappeared when animals were retested while simultaneously exposed to toluene. To date, relatively few studies have evaluated how drug administration during testing sessions affects the expression of preference (or aversion) following place conditioning and most studies utilize only a drug-free testing condition. For example, relative preference produced by two different doses of amphetamine was only expressed in animals tested under the influence of the conditioning drug (Laviola and Adriani, 1998). Interestingly, in the report by Bespalov et al. (1999), a dose-dependent increase in the time spent in the morphine-paired compartment was only observed when the same doses were used for the training and testing sessions. This finding contrasts with the present study in which not only did the testing in the presence of toluene exposure (1895 ppm) not enhance the preference score, but instead actually decreased the preference for the toluene-paired compartment. The basis for this decreased expression is not known at the present time. One possible explanation is based on the report that the N-methyl-D-aspartate (NMDA) antagonist dizocilpine interferes with the expression, but not the acquisition, of bromocriptine-induced sensitization (Carlezon et al., 1995) and that NMDA antagonists

in general interfere with the expression of learned drug-environment associations (Bespalov et al., 2000). Experimental evidence that toluene acts as a noncompetitive NMDA antagonist *in vitro* and *in vivo* (Cruz et al., 1998) leads to the hypothesis that testing during toluene exposures extinguishes cue-induced associations.

It should also be noted that the tests with toluene exposure occurred after tests in the toluene-free condition without any intervening training. Thus, there is an order effect, which confounds interpretation of these test session differences. For example, the loss of the conditioned place preference in the second test session could simply reflect extinction of the toluene-environment association. However, more research is needed to clarify the potential mechanism of state dependency in toluene-induced conditioned place preference.

Locomotor data indicate that the number of beam breaks during the test session was significantly lower when the animals were exposed to the training concentration of 1895 ppm as compared to their locomotion during the drug-free (air only) test. At first glance, this is not surprising, since toluene has been reported to increase spontaneous locomotor activity at lower doses and to decrease activity at higher exposure levels (3000-6000 ppm) in mice (Wood and Colotla, 1990). However, there is one important caveat that should be considered here. The decrease in locomotor activity induced by exposure to 1895 ppm toluene relative to air test conditions was observed only in the paired (treated) group of animals, but not in the control, toluene-naive group (Fig 2.1B). It appears then that the toluene-paired animals were more sensitive to locomotor suppressing effects of toluene than toluene-naive animals. This is consistent with the concept of sensitization, previously reported for other drugs of abuse (for review, see

Kalivas, 1992; Kalivas and Stewart, 1991; Kalivas et al., 1992) and for toluene itself (Beyer et al., 2001).

There is evidence that toluene has anti-anxiety effects in mice in the dose range of 1000-4000 ppm (Bowen et al., 1996; Lopez- Rubaclava et al., 2000). However, in the present study, acute toluene exposure increased stressful effects as observed by the increase in fecal pellet output (see Fig. 2.4). It could be hypothesized that, with repeated training sessions, animals habituate to the aversive effects, as evidenced here by the return to control values of fecal boli already by the second training session (Fig. 2.4).

To further the understanding of the abuse liability of other classes of chemicals, the reinforcing effects of inhaled acetone was studied. The experimental conditions were identical to the place preference paradigm used for toluene.

This is the first study in which a dose-response relationship was established using inhaled acetone in freely moving, behaving animals. The place preference results suggest place conditioning effects of inhaled acetone in the concentration range of 5,000-20,000 ppm; however, no correlation between preference score and the number of pairings was observed at the 10,000 ppm concentration. Thus, while inhaled acetone produced a marked decrease in locomotor activity similar to those reported using CNS depressants (Evans and Balster 1991; Bowen and Balster, 1998; Bowen et al., 1996) these doses and pairing conditions did not produce rewarding effects using the CPP assay.

In vivo brain pharmacokinetics studies of radiolabeled acetone and toluene support the notion that these two solvents are biologically unique. For example, ^{11}C-acetone possesses a markedly slower uptake into the brain and clearance from the CNS compared to ^{11}C-toluene (Gerasimov et al., 2002). Thus, it follows that their

behavioral effects would reflect this biological difference, since fast brain uptake and clearance is associated with a drugs reinforcing properties (Volkow et al., 1999).

In conclusion, these studies demonstrate that the conditioned place preference paradigm can be used to study abuse-related behaviors of inhalants. Because toluene and acetone share many other properties with other solvents (Balster, 1998), it should be possible to apply this paradigm to the study of other inhalants as well. Toluene's conditioned place preference effects were concentration related and occurred in a concentration range consistent with previous behavioral and neurochemical effects mediated by dopamine. The conditioned place preference model should be very useful for evaluating the abuse potential of various solvents and studying the behavioral and neural bases for their abuse-related effects.

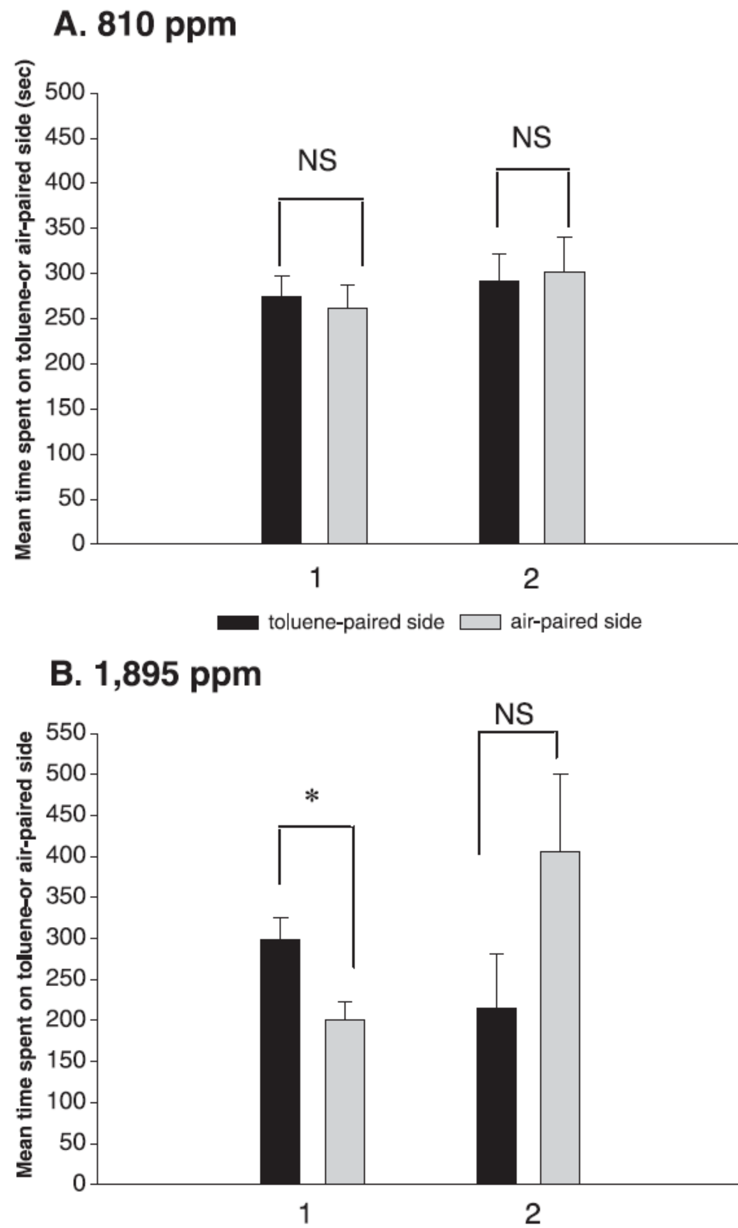


Fig 3.1 Results of tests for a conditioned place preference with two concentrations of toluene. Shown is the average time spent in the toluene or air-paired side on test day 1 (toluene-free) and on test day 2 (during exposure to the training concentration of toluene). (A) Animals conditioned with 810 ppm of toluene; (B) Animals conditioned with 1895 ppm of toluene. Data are means ± S.E.M. of eight rats per group. NS, not significant using a paired *t*-test, *p<0.05, **p<0.01. (adapted from Gerasimov et al., 2003)

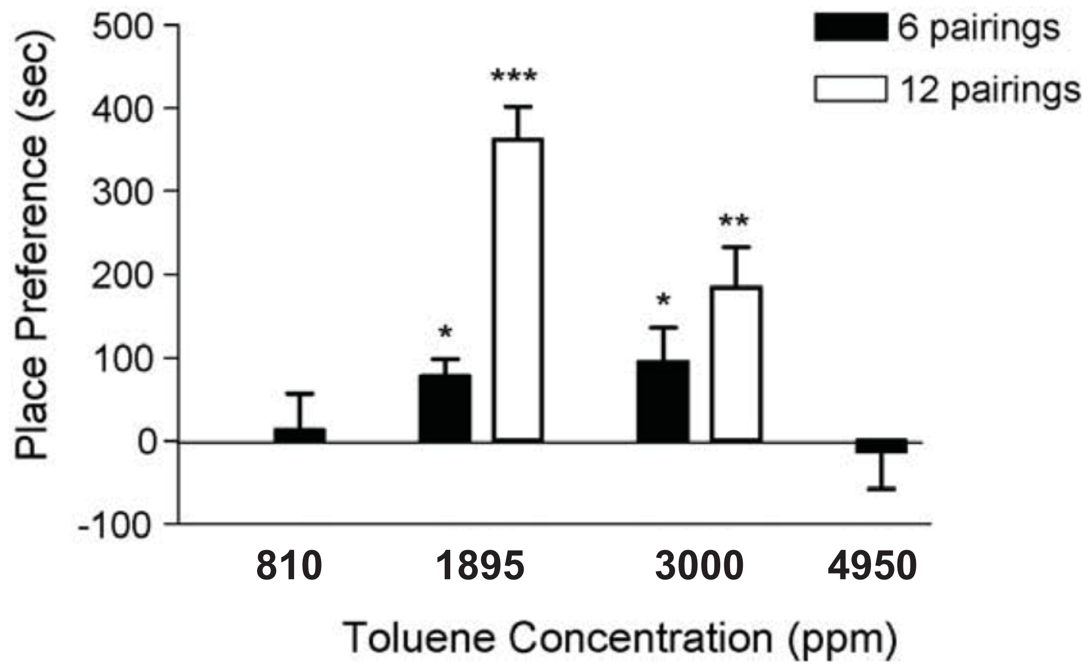


Fig. 3.2 Place preference following 6 or 12 conditioning pairings of inhaled toluene at the concentrations indicated. Data are means differences (±S.E.M.) between the times spent in the toluene- and air-paired sides of the CPP apparatus from 8 to 24 rats; * p<0.05, ** p<.001 vs. null hypothesis of zero preference (paired *t*-test). (adapted from Lee et al., 2006)

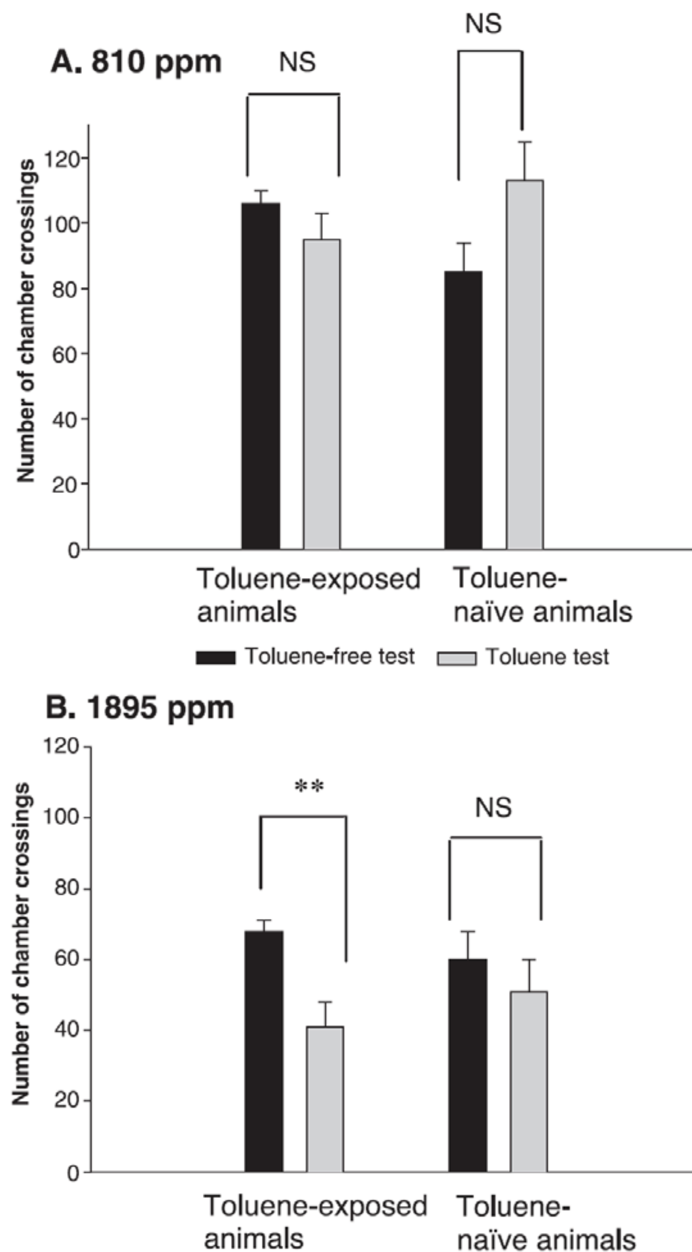


Fig. 3.3 Effects of two concentrations of toluene on locomotor activity during test sessions. Shown are the number of chamber transitions on test dayd 1 (toluene-free; left panel) and on test day 2 (during exposure to training concentration of toluene; right panel) for toluene-trained and toluene-naïve rats (A) Animals conditioned with 810 ppm of toluene; (B) Animals conditioned with 1895 ppm of toluene. Data are means ± S.E.M. of 8 rats/group. NS, not significant using a paired t-test, **$p<0.01$. (adapted from Gerasimov et al., 2003)

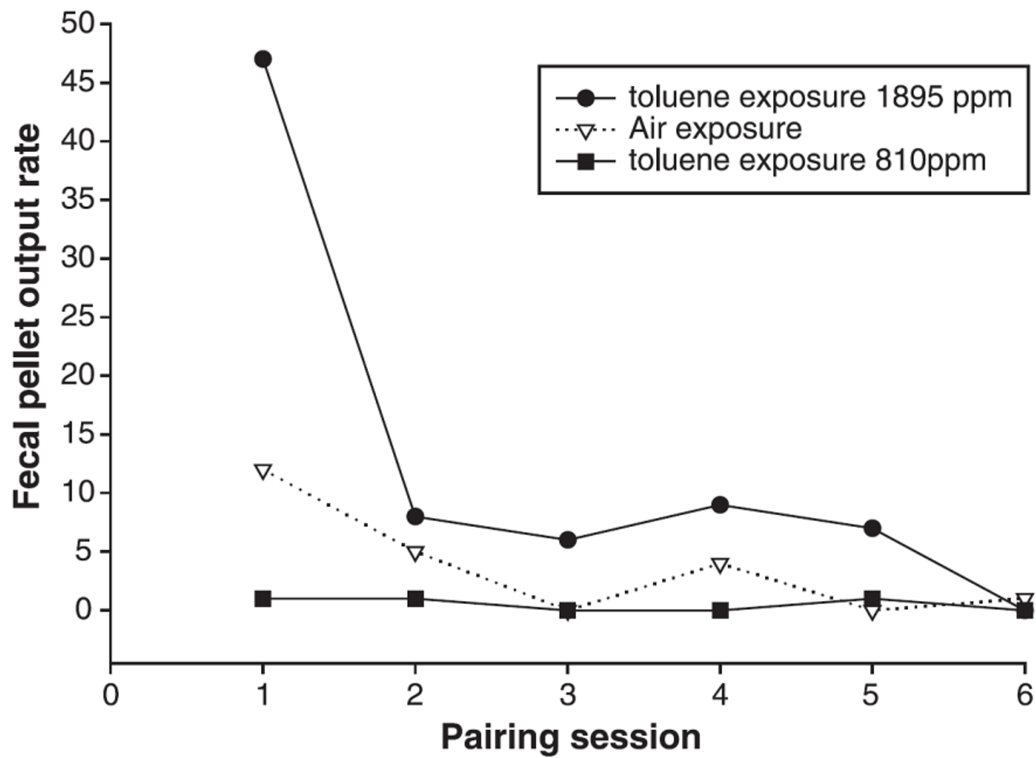
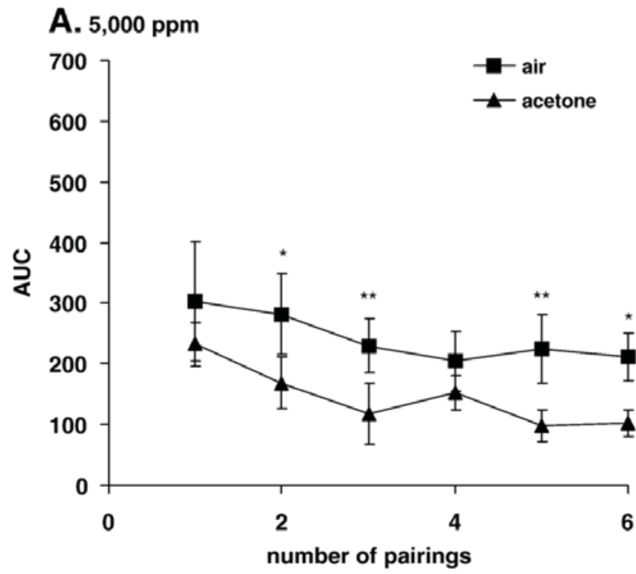


Fig. 3.4 Stress response to toluene vapors (810 or 1895 ppm) or air during the training sessions. Fecal pellets were collected after each conditioning session and counted. Data are a total number of pellets for each animal on a given day. (adapted from Gerasimov et al., 2003)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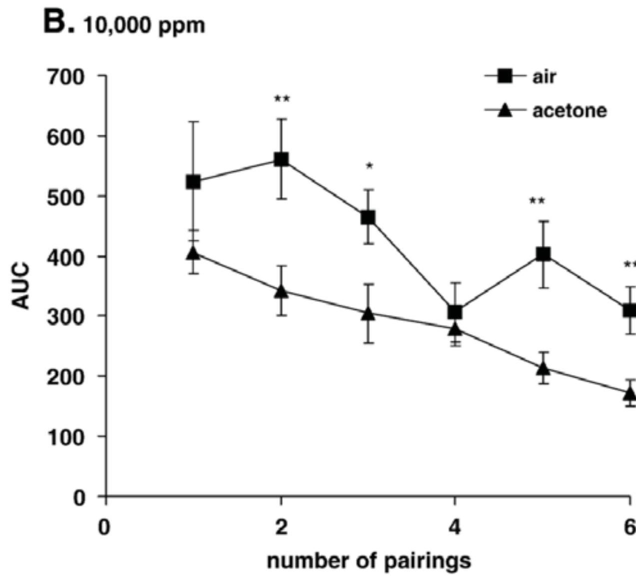
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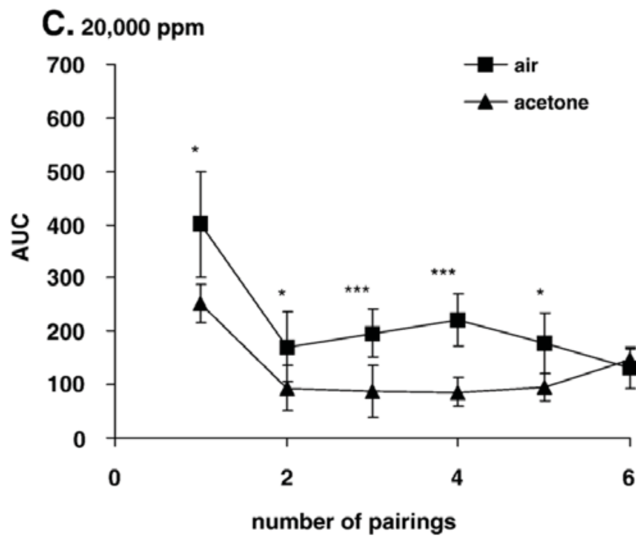
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Fig. 3.5 (A–C) Mean (±S.E.M.) area under curve (AUC) of horizontal movement counts during 1 h acetone inhalation period, separated into concentrations (5000, 10,000 and 20,000 ppm; n=8/group), and air during a 60-min period on alternate days for a total of 6 pairings. The three-way ANOVA yield a significant effect of treatment $F(1,287)=73$, $p<0.001$, exposure session $F(5,287)=23$, $p<0.001$ and concentration $F(2,287)=72$, $p<0.001$. Additionally, there was a significant interaction between exposure session and concentration $F(10,287)=2$, $p<0.05$. Lastly, A pairwise multiple comparisons (Student–Newman–Keuls Method) revealed significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, differences between each air session to each acetone session within the pairing regimen. (adapted from Lee et al., 2008)

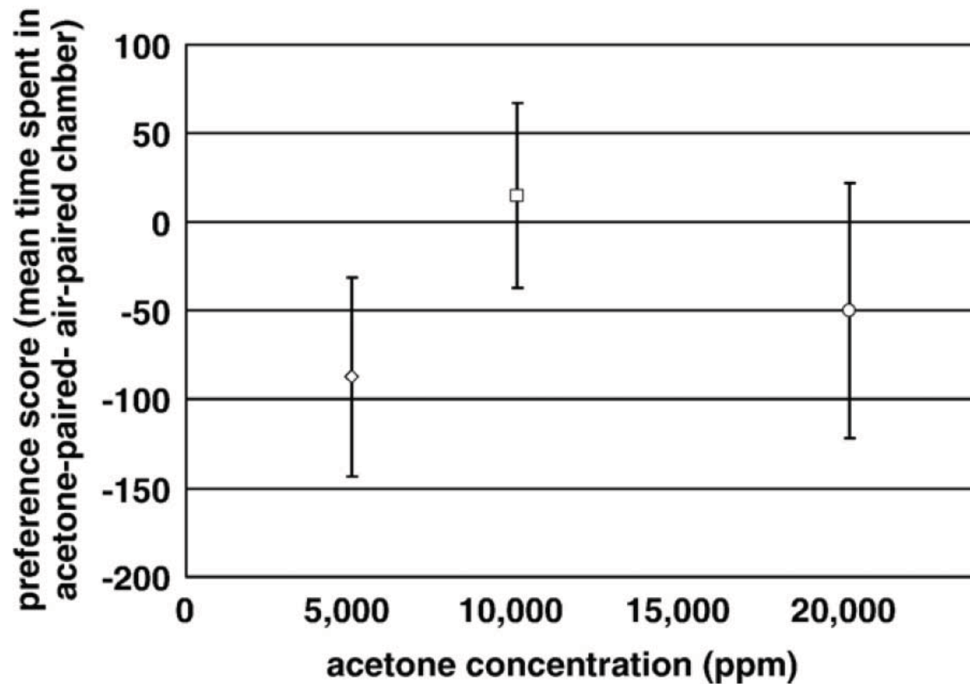


Fig. 3.6 Dose-effect relationship of acetone place preference conditioning. Preference scores (mean time spent in the acetone-paired chamber—mean time spent in the air-paired chamber) measured in an acetone-free environment (n=8/ group) after 6 pairing sessions for 1 h duration. The preference scores are plotted on the y-axis as a function of the acetone concentration (5000, 10,000, and 20,000 ppm) on the x-axis. A one-way repeated measures analysis of variance ANOVA comparison of preference score vs. concentration yielded a significant $F(1,47)=81$, $p<0.001$ dose-relationship effect. (adapted from Lee et al., 2003)

CHAPTER 4

Metabolic Function and White Matter Abnormalities in Toluene Exposed Rats: A Longitudinal Small Animal Imaging Study

Abstract

Solvent abuse is a significant problem among grade school children in the U.S. and worldwide. This also happens to be the critical age where the developing brain is most vulnerable to the effects of exogenous insult. Therefore, the effects of chronic toluene inhalation on brain structure and function were studied in animals exposed to a reinforcing course of toluene vapors using a conditioned place preference procedure. Here, changes in brain glucose activity were measured pre- and post-toluene conditioning using small animal PET (microPET) with (18)FDG. Next, structural changes, like white matter abnormalities, were examined pre- and post-toluene conditioning using small animal MRI (microMRI). A metabolic reduction of 20–40 % as measured by whole brain (18)FDG uptake was observed in toluene treated rats. The brain regions most affected were the frontal cortical regions. However, partial recovery of metabolic activity was observed in some regions, but not all, upon cessation. MicroMRI images revealed ventricular enlargement and white matter abnormalities compared to pre-toluene scans. These results are analogous to observations in human adult chronic solvents abusers who have severe white matter leukoencephalopathy along with many reports of enlarged ventricles. Small animal PET and MRI appear to be useful tools for examining the longitudinal brain effects of solvent abuse.

Introduction

Inhalants are widely available and easily accessible through household products. Several large scale national surveys have reported that 1/6 adolescents under the age of thirteen use inhalants to get “high” (Howard and Jenson 1999). Compare this to 1/32 adolescents aged thirteen and under who report trying cocaine, 1/56 who have used methamphetamine, or 1/77 that have used heroin (Cannon et al. 2008). It becomes clear that adolescent inhalant abuse is a serious problem.

Volatile solvents may pose an additional risk because of their highly lipophilic and toxic nature (Mattie et al. 1994), driving these compounds to organs rich in lipids, such as the brain. This poses a special risk to adolescents, who are in critical stages of development and may be more vulnerable to the effects of agents which target lipophilic brain regions. Because the primary solvent abusing population are children between the ages of 12 and 16 it is critical to determine whatever irreversible changes in brain structure and function are caused by early toluene exposure.

In fact, there is a growing concern that solvent abuse may result in irreversible structural damage to the nervous system leading to changes in behavior and neurological function [for review, see (Filley et al., 2004)]. Clinical studies have shown that, acute toluene intoxication produces a reversible syndrome characterized by encephalopathy and cerebellar ataxia, and toluene abusers who abstain from inhalant abuse show partial neurobehavioral recovery (Hormes et al., 1986). Further, chronic inhalation abuse induces a more severe white matter leukoencephalopathy characterized by a profound reduction in brain white matter regions (Filley et al., 2004; 1999) along with many reports

of enlarged ventricles. It has been hypothesized that these changes arise from a relative increase in the water content of the white matter caused by demyelination.

These physiological changes have been directly correlated with neuropsychological function and cognitive decline. However, only a handful of studies have attempted to gauge the physiological recovery from toluene abuse after cessation. Therefore, the effects of toluene inhalation on myelin integrity and glucose activity were measured longitudinally in rats using small animal imaging.

Previous efforts in our laboratory have been directed toward the development of an inhalation chamber that can assess the positive or negative reinforcing effects of inhaled solvents (Gerasimov et al., 2003; Lee et al., 2004), the conditioned place preference paradigm (CPP). First, animals were scanned at baseline (pre-toluene) using microPET with (18)FDG, a radioactive glucose analog. Then, a toluene conditioning ensued. This behavioral phase was followed by a series of scans (post-toluene) to assess the acute and long term metabolic effects of reinforcing toluene inhalation. The serial nature of these experiments provided the unique opportunity to assess the residual effects on brain function after the animals completed their conditioning protocol. In addition, white matter abnormalities in the brain were monitored in another group of animals using magnetic transfer magnetic resonance imaging (MT-MRI) (Quesson et al., 1998; 1997). MT-MRI provides a sensitive assay of myelination status in models of multiple sclerosis (MS), where the MT ratio (MTR) signal has been directly correlated with changes in the degree of myelination determined by Luxol Fast Blue (LFB) (Deloire-Grassinet al., 2000). Lastly, these imaging results were complimented by histopathology.

These studies demonstrate that toluene produces a marked effect on brain pathophysiology, and that this effect is regionally specific and recovers in some regions, but not others, after cessation.

Experimental Design

Subjects: All animals use procedures were in strict accordance with the National Institute of Health guide for the care and use of all laboratory animals and were approved by the local animal care and use committee. These studies utilized experimentally naïve, male, Sprague Dawley rats (Taconic Farms, Germantown, NY) that weigh 150–250 g and were given food and water *ad libitum*. Animals were housed in a vivarium with the temperature and humidity was kept relatively constant. All animals were housed in pairs and kept on a 12/12-light/dark cycle. Training and testing were conducted during the light cycle.

Animal PET: Animals were food deprived and brought to the microPET facility approximately 3 hours prior to scanning. Animals were injected intraperitoneally (i.p.) with 600–1000 μCi (18)FDG 50 min prior to anesthesia. The 50 min time period was chosen to be sufficient for equilibrium between (18)FDG in plasma and brain. After the uptake period, animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Each animal was positioned prone on the bed with the brain in the center FOV. Fully 3-D listmode data was collected in a single time frame of 600 s. One blood sample was taken at the end of the scan to measure whole blood glucose levels.

Animal PET Analysis: Images were reconstructed by a 3D maximum a posteriori (MAP) algorithm with 18 iterations and regularization parameter of 0.005, as previously described (Lee et al., 2006). A mean (18)FDG image was created in SPM2 (Statistical Parametric Mapping). Data from each animal was then coregistered to the mean (18)FDG image using a mutual information (MI) algorithm implemented with the Pixelwise Modeling (PMOD; Zurich, Switzerland) software package. The MLEM image pixel size

was 0.4 mm × 0.4 mm × 1.21 mm (*x*, *y*, *z*). A ROI template was developed in PMOD based on a digital atlas of the rat brain (Paxinos and Watson, 1997), or mouse brain (Paxinos and Franklin, 2007) as previously described (Lee et al, 2006). Briefly, the region of interest (ROI) template was developed in PMOD which included 10 regions; the frontal/prelimbic/cingulate cortex (FrA-PrL-Cg; with the center at ±0.9 mm lateral to bregma, *x*-axis; +1.6 mm anterior to bregma, *y*-axis; −3.1 mm below bregma, *z*-axis), primary motor cortex (M1; ±3.4, +1.3, and −3.1), sensory cortex (S1; ±4.3, −0.9, −4.6), auditory/temporal cortex (temporal, AuTeA; ±5.5, −5.3, −4.6), cerebellum (CB; ±1.9, −12.5, −6.0), caudate/putamen (CPu; ±2.2, +0.6, −5.6), thalamus (Thal; ±1.8, −3.2, −6.4), hippocampus (Hippo; ±2.4; −4.2, −4.0), midbrain (±0.2, −6.2, −6.3) and pons (±0.3, −8.5, −7.9). The mean volume of all ROIs was 0.86 cm^3 and the shapes were elliptical or round. Data were normalized for whole brain uptake and compared between datasets using $P < 0.05$ significance threshold.

Across subject variability was determined by Coefficient of Variable (CV = standard variation/mean). Statistical analysis included a two-way repeated measures analysis of variance (ANOVA), with ROI as one factor and age as the repeated measures factor. Statistically significant results were followed up using Student Neuman-Keuls post hoc tests. Lastly, for qualitative results, a mean image was created in Statistical Parametric Mapping 2 (SPM) software.

Results

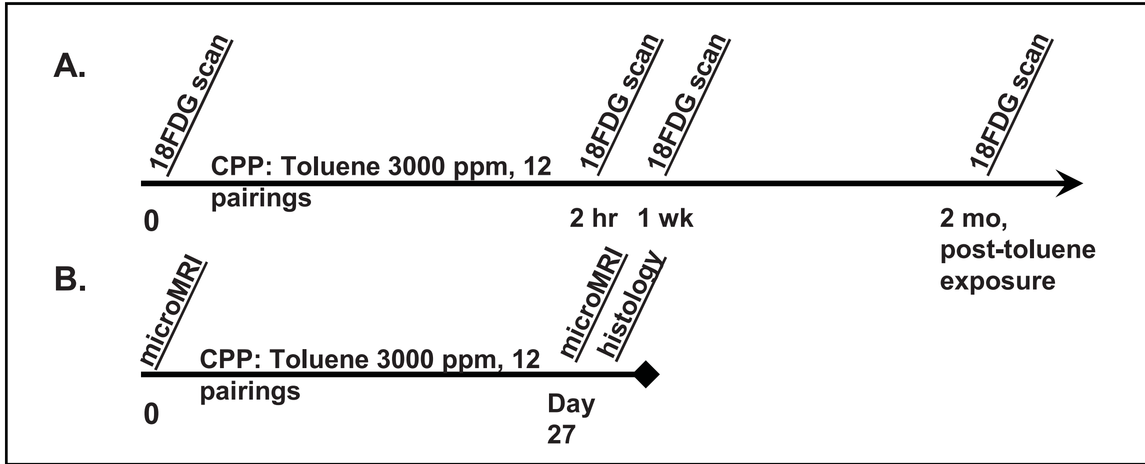


Fig. 4.1 Experimental design for combined imaging and behavior studies in animals (n=4 / per group) conditioned with 3000 ppm of toluene for 12 pairings.

Place preference results

In the preconditioning test, male Sprague Dawley rats (n = 4) showed no bias to either conditioning chamber with approximately equal time spent in each (black 234 ± 29 s; white 248 ± 4 s). After 12 pairings of 3,000 ppm, animals expressed a moderate to strong preference for the toluene-paired chamber (where moderate is defined by > 60 s and strong by > 90 s in the toluene-paired chamber). Cumulative time spent in the toluene-paired chamber was 295 ± 22 s, while a similar amount of time was spent in the air-paired chamber (207 ± 23 s).

Brain glucose, (18)FDG, uptake in toluene exposed rats

Alterations in brain glucose uptake were assessed using microPET. Rats received a baseline (18)FDG scan (pre-toluene) exposure. After a course of toluene CPP, the degree to which brain glucose activity was affected in several brain regions (post-toluene) were assessed at 2 hours, 1 week, and 2 months following toluene exposure. The scanning intervals and the experimental design are depicted in Fig. 4.1.

The microPET images depict qualitative results showing decline in (18)FDG uptake pre-toluene (Fig. 4.2a, left panel) to post-toluene inhalation (Fig. 4.2b, right panel). Quantitative changes in (18)FDG standard uptake value (SUV) were calculated from body weight, glucose, and blood concentrations of ^{18}F, using the formula: SUV=[(kBq/ml)/(Cb/g)*glu] where kBq/ml indicates kilobequerels per mL of brain tissue, Cb represents counts of whole blood (18)F per animal weight in grams and glu is the measured glucose level (mg/dl) at the time of the blood collection (at the end of the study). In the preconditioning scan (scan1; Fig. 4.3), the mean (±SEM) whole brain SUV was 33.9 ± 4.6. Two hours after the last toluene exposure (scan 2), the mean whole brain (18)FDG uptake was 21.4 % below pre-toluene values. One week after the last exposure (scan 3) whole brain uptake was reduced by 39.3 %, a reduction that was maintained for at least 2 months (scan 4; −33.7 %). In addition, two-way ANOVA revealed a significant main effect of scan ($F = 78.7$, $p < 0.001$) and a significant main effect of region ($F = 11.5$, $p < 0.001$), but the interaction between these factors was not significant ($F[3,15] = 0.31$, $p = 1.0$). This supports a toluene mediated global metabolic decline.

Recovery from this whole brain glucose deficit appears to be regionally specific. The evaluation of subcortical structures revealed improving metabolism over time (Fig. 4.3b), especially relative to cortical regions (Fig. 4.3a) where, except for the cerebellum, there was no move toward recovery. Thalamic metabolism showed a more prominent recovery at scan 4 than other subcortical regions, with a 6.1 % increase in (18)FDG uptake compared to the first post-toluene scan. (18)FDG uptake in the hippocampus also significantly increased between 1 week and 2 months cessation from toluene, indicated by a lack of significant difference from the initial scan (Fig. 4.3b). The cortical glucose deficit remained significantly lower than pre-toluene values 2 months after cessation of exposure (Fig. 4.3a). This glucose deficit was especially evident and significantly different at all time points scanned in the temporal, frontal and cingulate cortices.

Animals that received toluene gained less weight than the age-matched toluene-naïve animals, with a mean difference of 62 ± 15 g ($p = 0.061$), 53 ± 10 g ($p = 0.01$), and 75 ± 8.4 g ($p = 0.01$) at the second, third, and fourth scanning sessions (significant difference assessed using a Student's t test). Mean blood glucose levels were 155 and 145 mg/dl in the control and toluene-treated animals, respectively.

MT-MRI in toluene exposed rats

MT-MRI scans revealed enlarged ventricles in three of the four animals exposed to 12 pairings of 3000 ppm toluene vapors (Fig. 4.4A). White matter changes were observed with the greatest decreases in the medulla, corpus callosum, pons, and thalami (Table 4.1). On the contrary, hyperintensity was present in the frontal cortex,

sensory/motor cortex and the amygdala while the gray matter/white matter differentiation is preserved in the hippocampus and inferior collicus. Histopathology revealed similar results indicating enlarged ventricles (Fig. 4.4B).

Table 4.1. MTR MRI signal after 12 exposures of inhaled toluene in rats (n = 4)

	MTR MRI (% change)	
Regions of Interest	Mean	SD (standard deviation)
pons	-8.76	8.05
medulla	-16.84	8.85
midbrain	-9.59	15.13
thalamus	-9.17	8.87
inferior colliculus	3.24	18.14
superior colliculus	7.49	15.95
hippocampus	2.26	12.21
caudate	6.04	6.87
hypothalamus	6.31	9.80
frontal ctx	10.00	8.24
corpus callosum	-10.82	11.10
sensory/motor	11.54	11.51
amygdala	13.26	4.06
auditory cortex	7.22	7.78

Discussion

Toluene is a lipid-soluble aromatic hydrocarbon that is a common ingredient in household products that are widely available, inexpensive, and legal. These qualities promote solvent abuse in the adolescent population. Studies in adults revealed that acute toluene exposure produces mild dysfunction of the peripheral nervous system (Filley et al., 2004) and reversible neurological syndrome characterized by encephalopathy and cerebellar ataxia (Hormes et al., 1986) while chronic toluene abuse induces a profound reduction in brain white matter along with enlarged ventricles (Filley et al., 1990).

Because the CNS is highly organized on a structural and functional basis, it might be expected that the distribution of toluene, due to its lipophilicity, may regionally affect brain function. In fact, PET studies have demonstrated that the differential uptake and distribution of radiolabeled solvents (^{11}C-toluene, ^{11}C-acetone, and ^{11}C-butane) among various body organs can be largely attributed to factors like tissue lipid content and blood perfusion rates (Gerasimov et al. 2005). These same factors could be expected to affect toluene distribution within different regions of the brain. For example, Gospe and Calaban (1988) suggested that many of the neurological signs induced by toluene intoxication could be attributed to specific disturbances in brainstem and cerebellar outflow, and that this could be partially attributed to the accumulation of toluene in the lipid-rich regions of the pons and midbrain. Similarly, pioneering MRI studies (Filley et al. 1990; Rosenberg et al. 1988) provided evidence of a diffuse cortical atrophy accompanied by cerebellar and brainstem alterations in chronic solvent abusers. Moreover, a loss of differentiation between gray and white

matter throughout the CNS was attributed to toluene-induced changes in myelin integrity (Filley et al. 2004).

If the high degree of lipophilicity associated with toluene is a factor in determining regional brain uptake, then these high lipid target regions may be functionally affected the most. Several investigators have determined that regions with high lipid content in the rat brain are the pons, midbrain, thalamus, and cerebellum while regions with lower lipid content are the hypothalamus, hippocampus, and cortex (Ameno et al. 1992; Gospe and Calaban 1988; You et al. 1995). In this present study, MT-MRI scans in toluene exposed adolescent rat brains revealed regional hypointensity (Table 4.1) that agrees with clinical findings of myelin damage in chronic adult solvent abusers (Filley et al. 2004). These results were confirmed in brain tissue sections Weil stained for myelin which also revealed ventricular enlargement in three of the four animals scanned (Fig. 4.4).

Moreover, toluene exposure produced a global reduction (20–40%) in whole brain (18)FDG uptake (Fig. 4.2). Analysis of region-specific effects indicates that the subcortical regions are particularly sensitive to toluene-induced changes in (18)FDG uptake. Specifically, 2 hours after toluene exposure (Scan 2) the most significant decreases in (18)FDG uptake were the frontal and temporal cortices followed by the caudate/putamen and midbrain regions compared to pre-toluene scans. One week after cessation from toluene (Scan 3), a global reduction was observed in all brain regions and the effects were generally evident 2 months after last toluene inhalation (Scan 4). However, (18)FDG uptake as a measure of glucose activity was restored in the temporal cortex, hippocampus, pons, and cerebellum 2 months after cessation.

Thus, the pattern of altered (18)FDG uptake bears some correlation with the pattern of MTR hypointensity, but regions with lower lipid content such as the frontal and temporal cortices also appear to be significantly affected. The degree to which the regional distribution and mechanism of action of toluene and other abused inhalants are mediated by a receptor- or enzyme-driven mechanism is an area of active investigation (Balster 1998).

The present studies indicate that the frontal cortical brain regions appear more susceptible to the long term consequences of toluene exposure than striatal regions. This agrees with evidence that some behavioral effects of toluene can be mediated by influencing prefrontal cortex (PFC) dopamine (Riegel et al. 2003; Riegel and French 1999), glutamate (Cruz et al. 1998), or GABA transmission (Lee et al. 2004; Riegel and French 1999).

These studies demonstrate that toluene produces a marked effect on brain metabolic function, and that this effect is regionally specific and recovers in some regions, but not others, after cessation. Additionally, white matter abnormalities and ventricular enlargement were also observed in chronic toluene exposed animals. Future imaging studies will be conducted throughout the life span of the animals to examine further the long term consequences and recovery of toluene abuse.

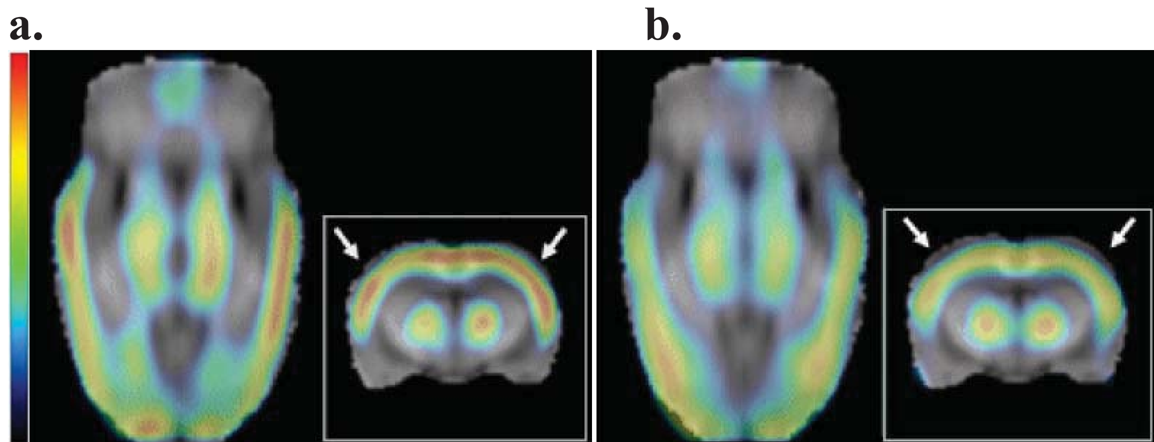


Fig. 4.2 A mean microPET image showing (18)FDG uptake in rodents pre and post toluene expoure. Transverse and coronal (insets) image slices are shown at the level of the rat striatum. a) (18)FDG baseline scan and b) (18)FDG uptake after toluene inhalation (12 exposures; 3000 ppm). A global reduction of (18)FDG uptake was observed after toluene exposure especially in cortical regions (arrow). (adapted from Schiffer, Lee et al., *unpublished data*)

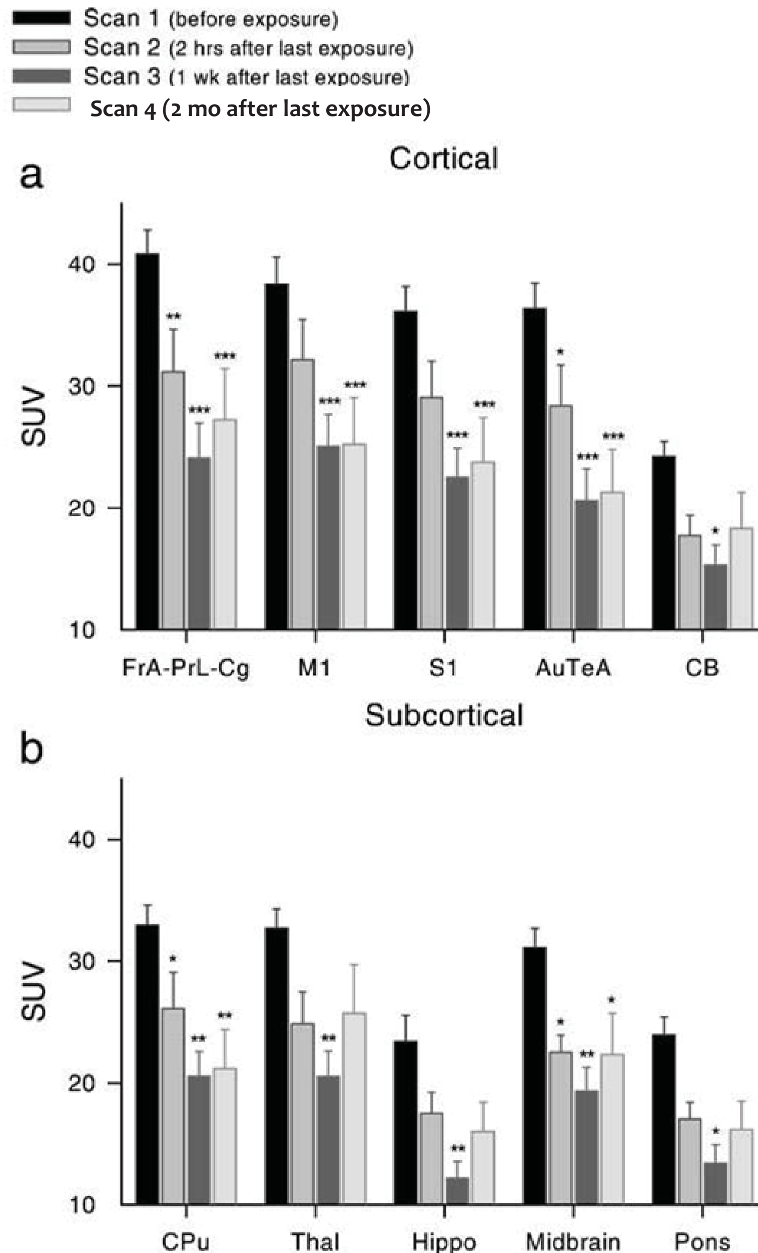


Fig. 4.3 Regional variation in (18)FDG uptake after a conditioning course of inhaled toluene (3,000 ppm). Values represent mean standard uptake value (SUV)±SEM. (18)FDG uptake occurred while animals were awake and in their home cages, followed by light anesthesia and a 10-min microPET acquisition. The ROI template included 10 regions based on a functional atlas of the rat brain (Paxinos and Watson 1986); ROIs: frontal/prelimbic/cingulate cortex (FrA-PrL-Cg), primary motor cortex (M1) sensory cortex (S1), auditory/temporal cortex (AuTeA), cerebellum (CB), caudate/ putamen (CPu,), thalamus (Thal), hippocampus (Hippo), Midbrain and Pons. The mean volume of all ROIs was 0.86 cm^3 and the shapes were elliptical or round. Significant differences from pretoluene (18)FDG uptake (scan 1) are indicated with *$p<0.05$, **$p<0.01$, and ***$p<0.001$. (adapted from Schiffer, Lee et al., 2006)

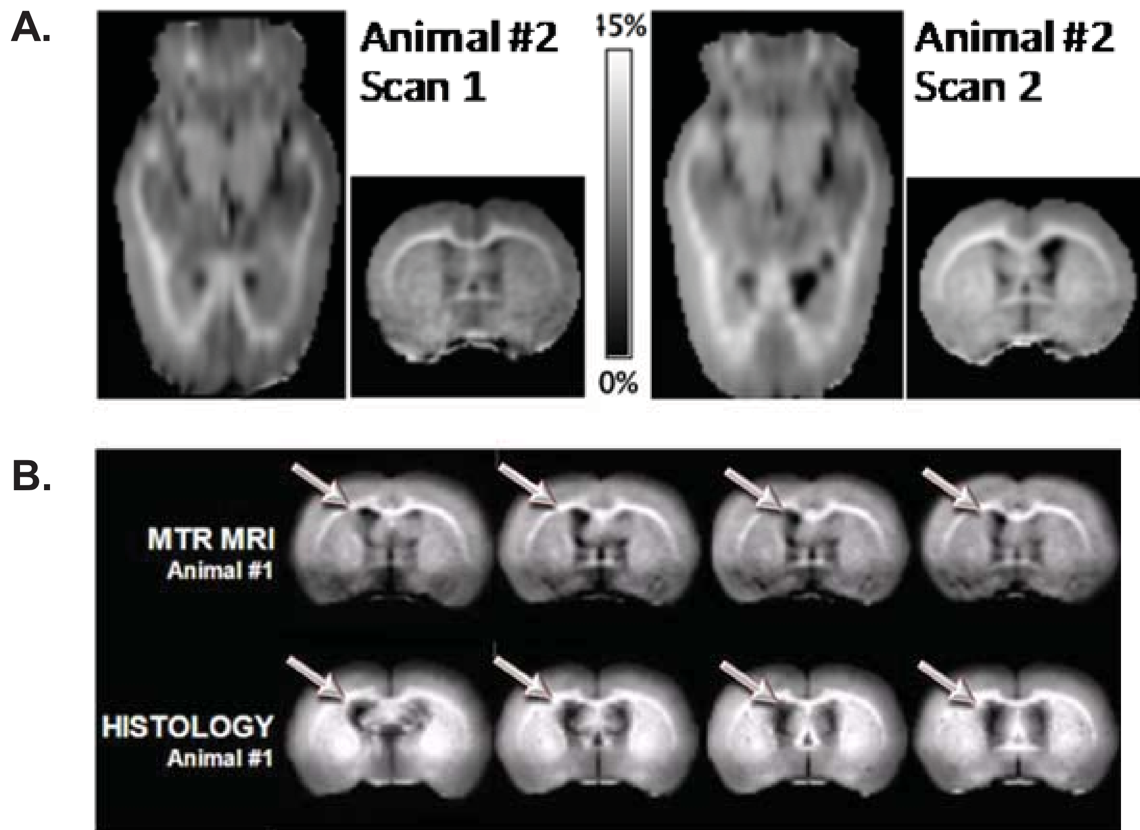


Figure 4.4 Representative MRI images showing ventricular enlargement. A) Animal #2 Scan1 at baseline; the same animal after toluene exposure (12 pairings with 3000 ppm), Scan2. B) coronal image slices showing ventricular enlargement (arrow) in MRI image slices and reproduced in tissue sections.Ventricular enlargement was observed in three of the four animals scanned, however across animals it was not always localized to the same hemisphere, as demonstrated here. (adapted from Schiffer, Lee et al., *unpublished data*)

CHAPTER 5

Targeting the Neurochemical Mechanisms Underlying Solvent Abuse

Abstract

Toluene is the main intoxicant in many of the products that are inhaled by school children. Although inhalant abuse is now being recognized as a worldwide problem, solvents are currently the least studied drugs of abuse. Therefore, we examined the central reward circuit, the mesolimbic dopaminergic system, using a dopamine receptor ligand, [(11)C]raclopride and microPET to measure alterations in toluene-induced dopamine synaptic function. In addition, metabolic activity was measured using (18)FDG, an analog of glucose, during a drug-seeking behavioral test to understand which regions of the brain are important for toluene reinforcement. Toluene caused a 13 % reduction in [(11)C]raclopride binding. Enhanced regional brain glucose activity correlated with an increase in the animal's drug-seeking behavioral score. These studies provide support for the use of microPET as a tool for monitoring brain and behavior relationships.

Introduction

Solvent abuse, especially in adolescence, has been reported to occur world-wide (Flanagan and Ives 1994; Kozel, 1995). However, relatively little is known about the underlying molecular and cellular mechanisms of action through which these substances produce their effects in the central nervous system (CNS). Therefore, these studies were performed to understand the neurochemical mechanisms underlying toluene abuse.

The recent commercialization of a new generation of high-resolution small animal PET cameras (microPET) has been instrumental to the use of PET in basic research and new drug development. PET has temporal resolution in the nanosecond range and spatial resolution of ~ 2 mm. It can be used to monitor whole body distributions of drugs to estimate kinetic parameters and to evaluate receptor occupancy *in vivo*. Further, high-resolution PET can help bridge decades of biological research in laboratory animals with clinical research. For example, microPET imaging of the rodent brain has been extensively used to study dopaminergic function in animal models of brain diseases (Hume et al. 2004; Dewey et al. 1992) including Parkinson's disease (Gasser et al., 1998), schizophrenia (Wong et al. 1986; Wong et al., 1986; Seeman, 1987), and drug abuse (London et al, 1999; Childress et al. 1999).

The utility of using animal PET cameras and specially designed radioligands to study dopaminergic function in the rodent brain was first investigated a little more than a decade ago (Hume et al., 1996). This and more recent studies have demonstrated that animal PET can give reproducible data that are highly consistent with more established ex vivo and *in vitro* techniques

(Logan et al., 1991). The dopamine receptor radioligand, [(11)C]raclopride, competes with endogenous dopamine for the dopamine type 2 (D2) receptor sites on the postsynaptic membrane. Therefore, its displacement can be used as an indirect assay of synaptic dopamine release in response to drugs of abuse (Gatley et al. 1997; Logan, Dewey et al. 1991). Alternatively, when dopamine is depleted with drugs like reserpine or α-methyl-para-tyrosine (AMPT), [(11)C]raclopride can be used to measure D2 receptor density in the striatum, a D2DR-rich region (Smith et al., 1988). Another commonly used radiotracer is (18)FDG, an analog of glucose (Reivich et al., 1979). This tracer is one of the few radioligands approved by the U.S. Food and Drug Administration (FDA) for diagnostic purposes. The mechanism of (18)FDG utilizes the basic principles of glycolysis. Cells take up glucose or (18)FDG and is phospholyated by hexokinse. However, unlike glucose-6-phosphate, (18)FDG-6-phosphate can not further metabolize and gets effectively trapped intracellularly.

Both radiotracers have been used extensively to study the pharmacokinetics and pharmacodynamics of drugs of abuse. These studies have shown that fast striatal spike and rapid release are associated with their rewarding effects in humans, as reflected by self-report of the intensity of euphoria or 'high' in humans (Gatley et al. 2005; 1997). Moreover, chronic cocaine abuse produces prolonged effects on energy demand in specific brain regions, particularly frontal and striatal regions. Additionally [(11)C]raclopride binding site density is significantly reduced after prolonged abuse (Volkow et al., 1993; 1996). Taken together, it follows that impairments in striatal dopamine function might be related to metabolic deficits in distant regions, namely the frontal cortex.

Therefore, the aims of the present study were to investigate the effect of toluene on dopamine transmission, using [(11)C]raclopride displacement as a measure of synaptic dopamine release. In addition, (18)FDG brain uptake was correlated with the animal's preference for toluene-related environmental cues.

Experimental Design

Subjects: All animals use procedures were in strict accordance with the National Institute of Health guide for the care and use of all laboratory animals and were approved by the local animal care and use committee. These studies utilized experimentally naïve, male, Sprague Dawley rats (Taconic Farms, Germantown, NY) that weigh 150–250 g and were given food and water *ad libitum*. Animals were housed in a vivarium with the temperature and humidity was kept relatively constant. All animals were housed in pairs and kept on a 12/12-light/dark cycle. Training and testing were conducted during the light cycle.

Animal PET: Imaging was performed using an R4 tomograph (CTI, USA), which has a transaxial resolution of 2.0 mm full width at half maximum (FWHM). Animals were anesthetized with ketamine and xylazine (10:1) and catheterized for lateral tail vein injection of ~798 uCi [(11)C]raclopride corresponding to an injected raclopride mass of ~1.5 nmol/kg. Each animal was positioned prone on the microPET bed with the brain in the center field of view (CFOV). Fully 3-D listmode data was collected for 60 mins starting at time of injection and histogrammed into 24 time frames (6 × 10 s, 3 × 20 s, 8 × 60 s, 4 × 300 s and 3 × 600 s). The resulting sinograms were reconstructed using Expectation-Maximization (EM) algorithm for maximum likelihood (ML) after randoms subtraction. Scattered events were accounted for using a two tail-fit algorithm (Alexoff et al., 2003).

(18)FDG Uptake during Drug-Seeking Behavior: Sprague–Dawley rats received a baseline PET (18)FDG scan. Rats were separated into two groups: toluene group and saline group. Rats in the toluene group were alternately administered toluene vapors

(3000 ppm, 12 pairings) or saline in distinct environments using a behavioral model of cue-induced reward (Conditioned Place Preference), whereas rats in the saline group were administered strictly with saline in the two distinct environments. Rats received a second (18)FDG scan during the place preference test. On the (18)FDG scan day (24 h after the final pairing session), rats were placed in the middle acclimation compartment of the CPP apparatus, injected with (18)FDG, and given access to all compartments during the 45 min uptake period. Time spent in each environment was electronically recorded and rated with a computerized automated system. Rats were then anesthetized and scanned for 10 min. (18)FDG data were normalized to stereotaxic space and proportionally scaled to the global mean. Voxel-wise analysis was implemented in SPM2 and verified by Region of Interest (ROI) regression analysis performed using PMOD, where the main factor of interest was preference score (time in cocaine-paired compartment minus time in vehicle-paired compartment).

Animal PET Analysis: For the [(11)C]raclopride studies, the binding potential (*B*max/*K*d) was computed using the graphical analysis method without blood sampling (Logan et al., 1996). The BP, which can be derived from the distribution volume ratio (DVR) using the equation BP = DVR-1, was used for comparing data sets. This metric is the ratio of the receptor (target protein) concentration to the dissociation constant, and has been shown to be a reproducible measure of dopaminergic function *in vivo* (Gatley et al. 1997). Images were reconstructed by a 3D maximum a posteriori (MAP) algorithm with 18 iterations and regularization parameter of 0.005, as previously described (Lee et al., 2006).

Results

In rats, [(11)C]raclopride levels in the striatum and cerebellum reached equilibrium ~ 30 mins post i.v. injection. The distribution volume ratio (DVR), the steady state ratio of striatum to cerebellum [(11)C]raclopride, was unchanged at approximately 3.3 during the equilibrium period. When 20 mg/kg of toluene was introduced, the DVR decreased to 3, a 10 % change from equilibrium levels. To compare datasets, the binding potential (BP) was computed using the graphical method without blood sampling (Logan, Dewey et al., 1991) which can be derived from the DVR using the equation BP = DVR-1. Determinations of BP show toluene-induced displacement of [(11)C]raclopride by ~ 13 % (Fig. 5.1).

Animals conditioned with 2000 ppm of inhaled toluene for 6 pairings spent significantly more time in the toluene-related environment when given access to both the toluene-paired and air-paired compartments. Locomotor activity was also elevated during toluene-paired sessions, as previously studied (Gerasimov et al., 2002). The microPET imaging results show that compared to the baseline scan (before toluene exposure) rats that expressed a preference to the toluene-related environment had increased global (18)FDG brain uptake, as well as regional activations in the prefrontal cortex, limbic regions, and thalamus (Fig. 5.2).

Discussion

Solvent abuse can be defined by the recreational inhalation of chemical vapors, such as nitrates, ketones, aliphathic and aromatic hydrocarbons (Bruckner and Peterson 1977). A recent survey showed that 23 % of middle school and high schoolers in the U.S. have experimented with inhalants. At this early age, solvents will most likely be the first drugs children encounter. These early exposures predispose them to abuse harder drugs later in life. The incidence of abuse is clear; however, there has been remarkably little literature to elucidate the underlying molecular mechanisms of action through which these solvents produce their rewarding effects.

The main intoxicant in most abused solvents, toluene, was thought to act in a non-specific manner through membrane lipids due to its high lipid solubility. More recent reports indicate that toluene and classical non-volatile CNS depressants like benzodiazepines and barbiturates elicit similar behavioral responses (Evans and Balster 1991). These latter drugs act directly with ionotropic receptors and ion channels. More recent reports show that toluene modulates the mesolimbic dopamine reward circuit, specifically where the dopamine projections originate, the VTA (Rigel et al., 2007). Taken together, it appears that several neurontransmitter systems may be taking a role in the abuse liability of toluene.

The well established role of NAc dopamine release as the principle element generating reward-related behaviors has been thoroughly examined at Brookhaven National Laboratory (Upton, NY). The neurotransmitter, dopamine, is necessary for survival. It plays a key role to the response to external and internal stimuli, motivation, reward, and locomotor activity, among others. However, the imbalance of dopamine

levels is a key hallmark of many psychiatric diseases, like Parkinson's, schizophrenia, and addiction. Microdialysis studies have revealed the profile of NAc dopamine release in response to natural and exogenous rewards. For instance, natural rewards like food and physical handling release extracellular dopamine ~ 100–150 % over baseline levels. However, drugs of abuse elevate basal dopamine levels several hundred percent over natural rewards.

Analogous studies have been performed in humans using PET imaging which measures dopamine release by the displacement of [(11)C]raclopride, a dopamine receptor (D2) antagonist. Thus, the less [(11)C]raclopride is bound to the receptor, the higher the release of dopamine in the synapse (Gatley et al. 1997). This inverse relationship has allowed us to map out the profile of [(11)C]raclopride displacement of many drugs of abuse. Further, in non-human primates and rodents, drugs abused by humans increase dopamine release proportionally with their drug-seeking behaviors (CPP, self-administration) or locomotor activation.

The results show that toluene-induced dopamine release decreased [(11)C]raclopride binding by ~13 % (Fig. 5.1). This is in the range of binding that was expected since microdialysis studies show very little % change of nucleus accumbens dopamine (Gerasimov et al., 2002), analogous to changes in basal dopamine with natural rewards. There are several caveats to mention in the interpretation of these results. First, measurements were performed the first time the animal was exposed to toluene. From my research, toluene may have adverse effects initially; however, drug tolerance is reached after two exposures (Gerasimov et al., 2003). Also, these studies utilize different routes of administration, inhaled and i.v. (microdialysis and microPET, respectively). Also,

whether the concentration, length of exposure (40 mins), and the dose administered are clinically relevant needs to be addressed since solvent abusers administer drugs at smaller intervals, similar to smokers. Among many, a technical challenge I faced was the limit of sensitivity of the microPET camera and the [(11)C]raclopride method. Since toluene-induced accumbens dopamine release was minimal, my goal was to measure whether small changes in dopamine release can be seen in changes [(11)C]raclopride binding.

Further, to better understand brain behavior relationships using *in vivo* imaging methods, I combined an established animal model of inhalant abuse and simultaneous brain imaging to view changes in the brain as they are taking place. Basically, rats were confined to one distinct compartment where they received toluene vapors. The following day, they were confined to a second distinct compartment where they received air. After a number of pairing sessions (1 toluene exposure: 1 air exposure), the animals were placed in a 3rd neutral middle compartment that separates the two conditioning compartments. After 5 mins of acclimation in the middle compartment, the wall partitions were opened and the animal was given free access to all three-compartments. However, no drug was administered on the day of the test. The time spent in each compartment was measured.

I have shown that rats exhibit a preference for the toluene-paired compartment over the air-paired compartment, since the place preference test is performed without the positive stimulus (or negative for that matter). Thus, rats that spend more time in the drug-paired environment have made the association between a distinct environment (cue) and receiving the ‘positive stimuli’. Human PET studies have revealed the biochemical signature of “cue-induced craving”. That is, when addicts are shown drug paraphernalia or even an environment associated with drugs, glucose metabolism is elevated in brain

regions that are associated with memory and reward. Increased glucose metabolism is indicative of enhanced neural activity. Studies reveal marked activations in the amygdala and limbic regions.

Therefore, using the validated animal model for inhalant abuse, CPP apparatus, and experimental protocol, I set out to understand which areas of the brain are altered during drug-seeking behavior in rats. Following the last pairing session of inhaled toluene, a place preference test was given (as described above). However, before being placed in the middle chamber rats were administered (18)FDG. (18)FDG is a radiotracer analog of glucose which gets taken up readily by cells that are metabolically active (Reivich et al., 1979). Therefore, (18)FDG can enter the cell, like glucose, which then gets phosphorylated by hexokinase as the first step in glycolysis, Naturally, once phosphorylated, glucose-6-phosphate continues along the glycolytic pathway for energy production; however, FDG cannot enter glycolysis and becomes trapped intracellularly as FDG-6-Phosphate. Therefore, during the 40 minutes of the place preference test, the cells that are in high demand will readily take up glucose and (18)FDG. Rats were then anesthetized and scanned for 10 mins. Rats again expressed a strong preference for the toluene-related environment. Images from rats that were conditioned with toluene or air (controls) were preprocessed in PMOD, and then voxel-wise analysis (SPM) was performed to locate regional changes in (18)FDG uptake. Interestingly, compared to the air-paired controls, exposure to the toluene-related environment activated the frontal cortex and limbic regions, noticeably the striatum (Fig. 5.2). The pattern of brain activations in rats during exposure to a toluene-related environment resembles that of cue-induced craving in human subjects (Volkow et al., 2006). Further, the preference

scores of each animal were calculated (the mean difference between the time spent in the drug-paired minus air-paired compartments) to test the hypothesis that preference scores correlate with (18)FDG uptake. The results show that the animal's individual preference score correlates positively with whole brain uptake of (18)FDG. These microPET methods in freely moving rats support an experimental protocol in which i.p. injection of (18)FDG and a 10 min static scan can be used without altering the behavioral outcome of the study. Therefore, microPET and conditioned place preference can be useful tools for the study of brain / behavior relationships.

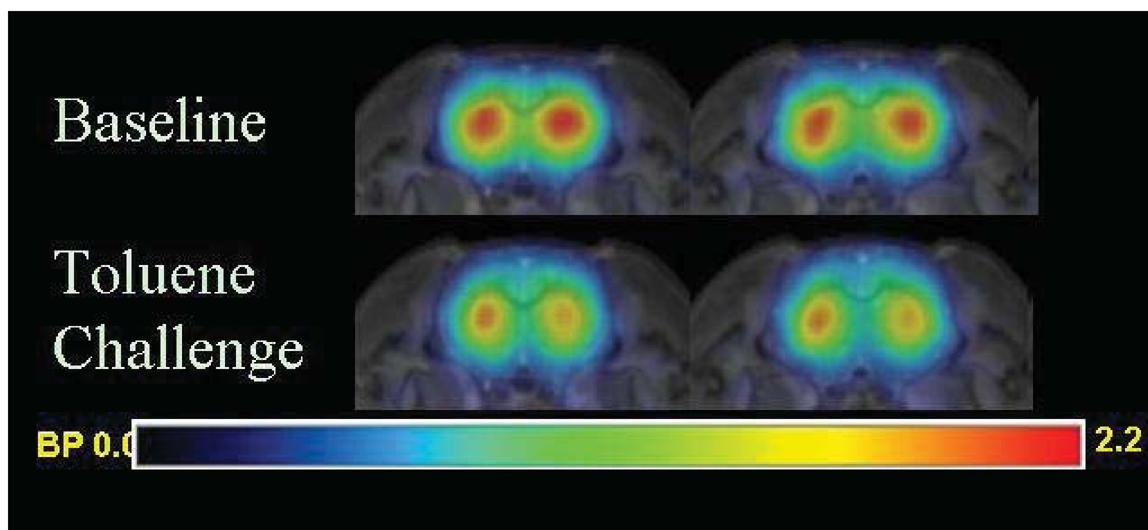


Fig. 5.1 A representative coronal image of [(11)C]raclopride binding in two Sprague-Dawley rats at baseline line (top panel), then after a toluene challenge. Rats (n=4) were injected with [(11)C]raclopride (~800 µCi ; corresponding to an injected raclopride mass of ~1.5 nmol/kg.). Once the levels in the striatum and cerebellum reached equilibrium (~ 30 mins) 20 mg/kg of toluene was injected i.v.. The steady state striatum to cerebellum ratio (specific to non-specific binding) was unchanged, approximately 3.3 at baseline. Upon toluene injection, the DVR decreased to 3, ~ 10 % change from equilibrium levels. Further, the binding potential (BP) which can be derived from the DVR using the equation BP = DVR-1, was use to compare datasets. Determinations of BP show toluene-induced displacement of [(11)C]raclopride by ~ 13 % which is reflected qualitatively in this figure (bottom panel). (adapted from Lee et al., 2004)

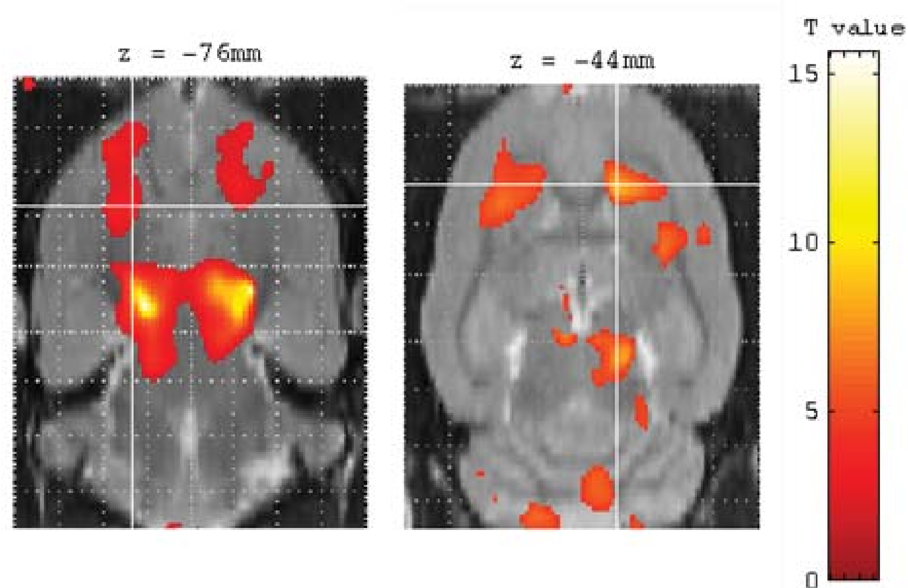


Fig. 5.2 Regional uptake of (18)FDG (~ 1 mCi) during a place preference test for toluene (6 pairings; 2000 ppm) in rats. Figure depicts an MRI transaxial view of the rat brain on the cortical (right panel) and subcortical (left panel) levels. This anatomical image is overlayed with significant activations (threshold of p<.001) of (18)FDG uptake during the place preference test (no drug administered). Therefore, the voxel-based analysis (colored clusters) depicts regions of activation during a cue-induced drug-seeking behavior. Activations in the prefrontal cortex, limbic regions, and thalamus were markedly different to air/air controls. (adapted from Schiffer, Lee et al., *unpublished data*)

CHAPTER 6

Dopamine Dysfunction in Type III Neuregulin 1 +/- Mice

Abstract

Rates of cocaine abuse are dramatically increased among people with schizophrenia. The considerable overlap in neurobiological disruptions thought to underlie each condition suggests that addictive behavior may represent a primary disease symptom of schizophrenia. Therefore, we investigated drug seeking behavior and alterations in the dopamine system in a genetically modified animal model of schizophrenia, the Type III *Nrg1* heterozygous mutant model. Type III *Nrg1* (+/-) and their wild type littermates (+/+) were paired with cocaine and/or vehicle in a conditioned place preference chamber equipped with infrared photocells for motor recording. Next, a small animal *in vivo* imaging instrument (microPET) and a radiolabeled D2 receptor antagonist, [(11)C]raclopride, was used to study the D2 receptor availability in Type III *Nrg1* (+/-) and wild type mice. Further, dopamine metabolism was measured using *in vivo* microdialysis in the nucleus accumbens. Type III *Nrg1* (+/-) mice expressed a significant ($p < 0.05$) cue-induced place preference to cocaine in an animal model of drug seeking behavior. Interestingly, the microPET data in Type III *Nrg1* (+/-) mice showed a direct correlation between D2 receptor availability and cocaine place preference. In addition, Type III *Nrg1* (+/-) mice had elevated DA metabolism *in vivo*. All together, our results indicate that genetic perturbation of the Type III *Nrg1* gene could affect brain function and vulnerability to cocaine abuse and is likely to be a valuable translational model for

understanding the neurobiological mechanisms underlying substance abuse and schizophrenia comorbidity.

Introduction

The prevalence of co-occurring schizophrenia and substance abuse in the US varies between 50–70 % (Gupta et al., 1996; Green et al., 2002), and exceeds 80 % when nicotine is included (Leonard et al., 2007; Lasser et al., 2000). These rates of substance abuse are much higher than those occurring in the general population, for example 20 % for nicotine. Among illicit substances, cocaine addiction is the most prevalent in schizophrenic patients, with 15–50 % lifetime abuse compared to 0.8 % in the general population (LeDuc and Mittleman, 1995). Cocaine abuse only complicates treatment options for schizophrenic patients resulting in poorer overall prognosis. Thus, investigations of cocaine abuse in schizophrenia could lead to more successful treatment options and favorable prognosis.

Given that most individuals with schizophrenia have substance abuse problems, reflecting self-medication to relieve negative symptoms, cognitive deficits, and or medication side effects. Additionally, genetic data from family and twin studies have implicated genetic factors to the development of schizophrenia, as well as genetic susceptibility to both disorders (Freedman et al., 2003; Ziedonis et al., 2005). However, the inconsistent concordance among monozygotic twins highlights non-genetic factors toward predisposition to schizophrenia. To circumvent interaction of genetic factors to environment factors, it becomes increasingly important to examine individual schizophrenia risk genes in relation to their specific neuronal endophenotypes.

Recently, a genome-wide scan of families in Iceland identified Neuregulin 1 () as a putative susceptibility gene to schizophrenia. An important approach to clarify the

functional roles of genes such as *Nrg1* is through the phenotype of mice carrying the *Nrg1* dysfunction. Interestingly, mutant mice heterozygous for *Nrg1* or *Nrg1* receptor, ErbB4, deficient mice show behavioral endophenotypes that overlap with humans with schizophrenia (Kellendonk et al., 2009). Type III *Nrg1* (+/-) mice exhibited altered prepulse inhibition of an acoustic startle (PPI), a test for preattentional mechanism which enables selective processing of external stimuli. Nicotine ameliorates PPI deficits, both in patients and in Type III *Nrg1* (+/-) heterozygous mice (Chen et al., 2008).

Much research has been undertaken in recent years to elucidate the biological functions of the NRG1 protein and to further the understanding of the pathogenesis of schizophrenia (Corfas et al., 2004). The NRG1 family of proteins has been implicated in synapse formation and function by regulating the expression of many neural transmitter receptors (Zhong et al., 2008).

The strongest candidates in this respect are the dopamine (DA), glutamate, GABA, and nAChRs systems (Mathew et al., 2007; Li et al., 2007; Laruelle et al., 2003; Leonard and Breese, 2000). Dopaminergic transmission is linked to schizophrenia. Antipsychotic actions of neuroleptic drugs are correlated with blockade of dopamine receptors (D2) (Farde et al., 1992). DA agonists, such as amphetamine both exacerbate symptoms in patients with schizophrenia, like psychosis and paranoia, and induce them in normal subjects (Laruelle et al., 1995). What's more, increasing numbers of studies have detected higher density of D2 receptors (Seeman, 1987) in schizophrenic patients than in the normal subjects, which has been reproduced in neuroleptic-treated and neuroleptic-naïve patients (Farde et al., 1992). Clearly, a dysfunction of brain dopamine is implicated in the neurobiology of schizophrenia.

Given that dopamine influences the properties of reward and motivation, the goal of these studies was to determine whether disruption of a schizophrenia risk gene altered drug seeking behavior and/or the dopamine system. To achieve this I measured cue-induced place preference, motor activity, postsynaptic dopamine receptor (D2) levels, and basal dopamine release in Type III *Nrg1* (+/-) mice and their wild type littermates.

Experimental Design

Subjects: Type III deficient *Nrg1* heterozygous mice ($Nrg1^{tm1.1Lwr}$) (Wolpowitz et al., 2000) and their wild type (C57/B6; Jackson Laboratory) littermates weighing between 25-29 g will be housed at Stony Brook University Division of Laboratory Animal Resources (DLAR) and transferred to Brookhaven National Laboratory (BNL) as needed. Animals will be maintained on a 12 h light/dark cycle and provided food and water *ad libitum*. All animal procedures were conducted during the light cycle and were in strict accordance with the National Institutes of Health guide for the care and use of all laboratory animals and were approved by the local animal care and use committee.

Conditioned Place Preference: During the conditioning phase animals received either compressed cocaine (20 mg/kg) or saline i.p. on alternate days for 20 min sessions. The conditioning chambers were cleaned with warm water following each animal's conditioning session. On the test day (the day immediately following the last cocaine exposure) mice were placed in the middle compartment for a 5 min acclimation period. Animals were then allowed free access to all three compartments for 20 min while their activity was monitored electronically.

Animal PET: Imaging was performed using an R4 tomograph (CTI, USA), which has a transaxial resolution of 2.0 mm full width at half maximum (FWHM). Animals were anesthetized with ketamine and xylazine (10:1) and catheterized for lateral tail vein injection of ~798 uCi [(11)C]raclopride corresponding to an injected raclopride mass of ~1.5 nmol/kg. Each animal was positioned prone on the microPET bed with the brain in the center field of view (CFOV). Fully 3-D listmode data was collected. The binding potential (Bmax/Kd) was computed using the graphical analysis method without blood

sampling (Logan et al., 1996). The BP, which can be derived from the distribution volume ratio (DVR) using the equation BP = DVR-1, was used for comparing data sets. This metric is the ratio of the receptor (target protein) concentration to the dissociation constant, and has been shown to be a reproducible measure of dopaminergic function *in vivo* (Gatley et al. 1997).

In Vivo Microdialysis: The mice were anesthetized with a ketamine/xylazine (10:1) cocktail; and placed in a standard stereotaxic frame using the standard adaptor for a mouse (Kopf). The scalp was incised (5-8 mm), the skull cleaned and two drill holes were made using a fine drill (0.7 mm diameter) for the fixing of the screws. Two microscrews were placed into the skull. Another hole (2 mm diameter) was drilled directly above the NAc, +1.5 mm anterior and ±0.8 mm lateral to bregma according to the mouse atlas (Paxinos and Franklin, 2007). A guide cannula 15 mm in length was lowered 4.8 mm below skull surface and secured with dental acrylic resin. After the resin was sufficiently dried, a membrane probe (1.0 mm; CMA Microdialysis) was positioned within the guide cannulae and perfused with artificial cerebrospinal fluid (148 mM NaCl/2.7 mM KCl/1.2 mM $CaCl_2$/0.85 mM $MgCl_2$, pH 7.4) at a constant flow rate of 1.0 µl/min. Samples were collected every 20 min and analyzed for dopamine (DA), homovanillic acid (HVA), and 3,4-dihydroxyphenylacetic acid (DOPAC) by high-performance liquid chromatography (HPLC) coupled to an electrochemical detection.

Immunoblotting: Striatal tissue was lysed in RIPA buffer (Thermo Fisher Scientific) supplemented with 10 mM DTT (Dithiothreitol), protease inhibitors (Sigma-Aldrich), and phosphatase inhibitors (Thermo Fisher Scientific). Lysates were be separated on 12 % SDS-PAGE gels and transferred to nitrocellulose filters. Filters were be blocked in 5 %

milk in 0.1 % Tween-20 TBS solution at room temperature for 2 h before overnight incubation in primary antibody solutions in 5 % milk in Tween-20 TBS solution at 4 °C (anti-D2DR [1:500; Santa Cruz Biotech, Inc.] and anti-GAPDH [1:15,000]).

Results

Table 6.1 Endophenotypes "shared" by patients and NRG1 (+/-) mice

Anatomical and Molecular Studies
increased ventricular volume*
reduced dendritic spine*
increased dopamine turnover and D2 receptors**
Behavioral Changes
impaired working memory*
sensory gating deficits*
altered reward, motivation, and reinforcement learning**
Pharmacological Responses
nicotine ameliorates sensory gating deficits*
show behavioral sensitization to psychostimulants**

*** (Chen et al., 2008)**
**** Lee DE, unpublished data**

Conditioned Place Preference

In order to test if reduced NRG1 significantly affects drug seeking behavior, I measured cocaine-induced place preference in Type III *Nrg1* (+/-) and wild type mice. Cocaine-induced place preference was tested under the unbiased CPP paradigm as previous described for other drugs of abuse (Tzschentke 2007). Type III *Nrg1* (+/-) mice (n = 5) conditioned with 3 pairings of 20 mg/kg of cocaine had an average preference score (mean time spent in drug-paired chamber - mean time spent in the saline-paired chamber) of greater than 100 s, whereas the average preference score for the wild type

mice after 3 pairings was 0 s ($p < 0.05$). Thus, reduced NRG1 signaling is associated with increased place preference to the cocaine-paired environment (Fig. 6.1).

Locomotor Activity

Motor activity is an indicator of sensitivity to reinforcing responses to psychostimulants (Suto et al., 2001; Piazza et al., 1989; Hooks et al., 1991a,b); therefore, I monitored the animal's locomotor activity during the CPP sessions to test whether the Type III *Nrg1* (+/-) and wild type mice differed in the development of locomotor sensitization response to cocaine. Mice were injected with cocaine (20 mg/kg i.p.) or saline before being placed in a CPP compartment for 20 mins. The locomotor activity was measured by the number of beam breaks of infrared photocells that lined the walls of the CPP apparatus. Type III *Nrg1* (+/-) mice had a 25 % increase in locomotor activity during three cocaine pairings compared to a 10 % increase in activity for their wild type littermates (Fig 6.2). No significant difference in beam breaks was observed in either cocaine-paired or saline-paired sessions in wild type mice. While there appears to be a slightly different basal motor activity in the two genotypes, previous studies conducted to characterize Type III *Nrg1* (+/-) mouse behavior (n>100) indicate that there is no difference in motor activity amongst this genotype (Chen et al., 2008).

Animal PET

Early reports on DA receptor changes after antipsychotic treatment showed an increase in striatal DA receptor number (Farde et al., 1992; Seeman, 1987). This finding has been replicated under a number of experimental conditions in neuroleptic-treated and

neuroleptic-naïve schizophrenic patients (Abi-Dargham et al., 2000; Laurelle et al., 1999). Here, I test whether Type III *Nrg1* (+/-) mutant mice have altered DA D2 receptor levels compared to wild type mice. To compare datasets, regions of interest (ROIs) were drawn manually on a MRI template which was then transferred to the coregistered microPET images. The ratios of [(11)C]raclopride binding in the striatum (binding to the D2 receptor target protein) to that in the cerebellum (non-specific binding) were calculated. There was a 13.5 % increase in [(11)C]raclopride binding in the striatum of *Nrg1* (+/-) mice compared to wild type mice ($p < 0.05$)., indicating a greater availability of D2 receptors (Fig. 6.3).

Immunoblot

Dopamine Type II receptor protein (D2DR; 98 kDa) levels in striatal homogenates were measured in Type III *Nrg1* (+/-) and wild type mice (n = 6 / group). Equal amounts of protein were resolved on a 12 % acrylamide gel, transferred to a nitrocellulose membrane and probed with a primary antibody for D2DR (Santa Cruz Biotechnology). Results were normalized to the respective immunoreactive bands of the loading control (GAPDH: 36kDA). Densitometric analysis revealed a 64 % increase in the band intensity in the Type III *Nrg1* (+/-) mice which neared significance ($p=0.09$) (Fig. 6.4). The combined results of the [(11)C]raclopride study and D2DR immunoblotting indicate an upregulation of striatal D2DR in Type III *Nrg1* (+/-) mice.

In vivo Microdialysis

Extracellular DA and metabolite (DOPAC and HVA) levels were measured using *in vivo* microdialysis in mice anaesthetized with a 10:1 mixture of ketamine and xylazine. 200 µm membrane probes (BSA) were inserted through a surgically implanted guide cannula into the nucleus accumbens shell (+1.5 AP, +0.8 ML, -4.8 DV) according to the Paxinos and Franklin atlas (Franklin, P. 1996). The dialysate was collected for a total of 150 mins. The first 90 mins of collection was considered the acclimation period. Fractions taken during the last 60 mins were analyzed for catecholamines according to HPLC methods as previously described. The total DA levels were not significantly different for wild type vs. *Nrg1* (+/-) (8.42 ± 1.70 ng/ml vs. 3.26 ± 0.72 ng/ml, respectively). Similarly, no change in total extracellular DA and metabolite (DA+DOPAC+HVA) concentrations were observed in wild type vs. *Nrg1* (+/-) (28.38 ± 1.56 ng/ml vs. 22.81 ± 2.80, respectively). However, the HVA / DA turnover ratio of 12.94 ± 1.05 in *Nrg1* (+/-) mice were significantly enhanced from the wild type ratio of 6.47 ± 0.64, $p < 0.02$. The DOPAC / DA ratios remained the same for wild type and *Nrg1* (+/-) mice (2.27 ± 0.35 vs. 2.85 ± 0.48, respectively; Fig 6.5).

Discussion

In the present study, Type III *Nrg1* (+/-) mice were examined for dopamine abnormalities. The main results are summarized in Table 6.1. I found that mice mutant for the schizophrenia risk gene, *Nrg1*, were more sensitized to the behavioral effects of cocaine (Fig. 6.1 and 6.2), had elevated D2 dopamine receptor availability *in vivo* (6.3) and in vitro (6.4), and increased basal dopamine turnover ratios (Fig. 6.5). Considering that dopamine dysfunction is a well established etiology of both schizophrenia and drug addiction, the alterations in dopamine transmission may explain the cocaine-induced drug seeking behavior and elevated locomotor activity observed in the Type III *Nrg1* (+/-) mice.

Further, working memory deficits are a central cognitive deficit seen in schizophrenic patients. Interestingly, mice over expressing D2 receptors showed impairments in working memory tasks and conditioned associative learning, both cognitive tasks requiring prefrontal cortex (PFC) (Kellendonk et al., 2009). Studies in Type III *Nrg1* mutant mice showed impairments in short-term and working memory tasks (Chen et al., 2008). Therefore, the increase in striatal D2 receptors in Type III *Nrg1* (+/-) mice, observed in the present study, is consistent with the established hypometabolism in the PFC, a neurobiological endophenotype shared by schizophrenic patients (Volkow et al., 1993). Moreover, it has been proposed that drugs of abuse induce hyperactive subcortical DA response that, in the presence of increased D2 receptors, intensifies both appetite and subjective high, due to "supersensitive" postsynaptic dopamine receptors. This data seems consistent with the known increased prevalence of substance abuse in schizophrenia, since an acutely enhanced DA response precedes sensitivity to addiction.

Next, presynaptic dopamine activity was assessed by measuring the major DA metabolite, homovanillic acid (HVA), in the nucleus accumbens. This approach is based on the understanding that extracellular HVA is related to DA release and metabolism and hence presynaptic activity (Bowers and Hoffman, 1984). The present study results show that DA turnover is higher in Type III *Nrg1* (+/-) mice than their wild type littermates. Moreover, I measured D2 receptor on multiple physiological levels to assess the levels of D2DR on the functional (surface receptors) and absolute (total protein) levels and found consistent increases across techniques. If that is the case, then the DA released is being turned over to HVA before it can bind to the postsynaptic receptor which would explain the increased in D2 receptor availability using microPET with [(11)C]raclopride. Interestingly, schizophrenic symptoms and plasma HVA levels are positively correlated in drug-naïve patients and in neuroleptic withdrawal patients who had worsening of symptoms which required hospitalization.

Further, the ultimate phenotype of any given molecular perturbation is defined by the behavioral response. Therefore, although we have thus far shown hyperactive dopamine transmission in Type III *Nrg1* (+/-) mice our next objective was to understand how these molecular alterations change responses to external stimuli. We have recently established an animal model for solvent abuse using the conditioned place preference paradigm (Gerasimov et al., 2003; Lee et al., 2004; 2006) which has been used to assess the motivational reward for many drugs of abuse (Tzschentke, 2007). Using this same three-compartment CPP apparatus we tested the hypothesis that Type III Nrg (+/-) mice would be more sensitized to the behavioral effects of cocaine in an animal model of drug seeking behavior. Our experiments gave us intriguing results. Type III *Nrg1* (+/-) mice

acquired a cue-induced place preference to the cocaine-related environment after 3 pairings of cocaine (20 mg/kg). Also, cocaine-induced locomotor activity was significantly higher in Type III *Nrg1* (+/-) than wild type mice. Taken together, our data indicate that Type III *Nrg1* (+/-) mice were more sensitized to the behavioral effects of cocaine which is consistent with the idea that the striatal D2 receptors in neuroleptic treated patients are in a supersensitive state so that the cocaine-induced DA release produced an enhanced drug seeking behavior.

NRG1 has multiple impacts on both ligand and voltage-gated ion channels. This is an active field of research which has focused on the dopaminergic system, GABAergic interneurons, and NMDA receptor transmission (Laruelle, 2000). Molecular changes in glutamate may be generally responsible for the cognitive dysfunctions, 'negative' symptoms, in the prefrontal cortex associated with working memory and learning (Krug et al., 2008; Li et al., 2007) and hyperactivity in mesolimbic/nigrostriatal dopamine (DA) may result in 'positive' symptoms (e.g. hallucinations and delusions (Laruelle et al., 2003), although no single neurotransmitter is clearly responsible for this complex brain disorder.

In conclusion, the present studies provide animal models to closely study dopamine-related brain behavior relationships. Future studies will use these models to explore the abuse liability of other abused substances, like volatile solvents, in the already genetically predisposed schizophrenic population. These findings warrant future investigations on the role of NRG1 signaling on the mesolimbic dopaminergic reward pathway that leads to cocaine sensitization.

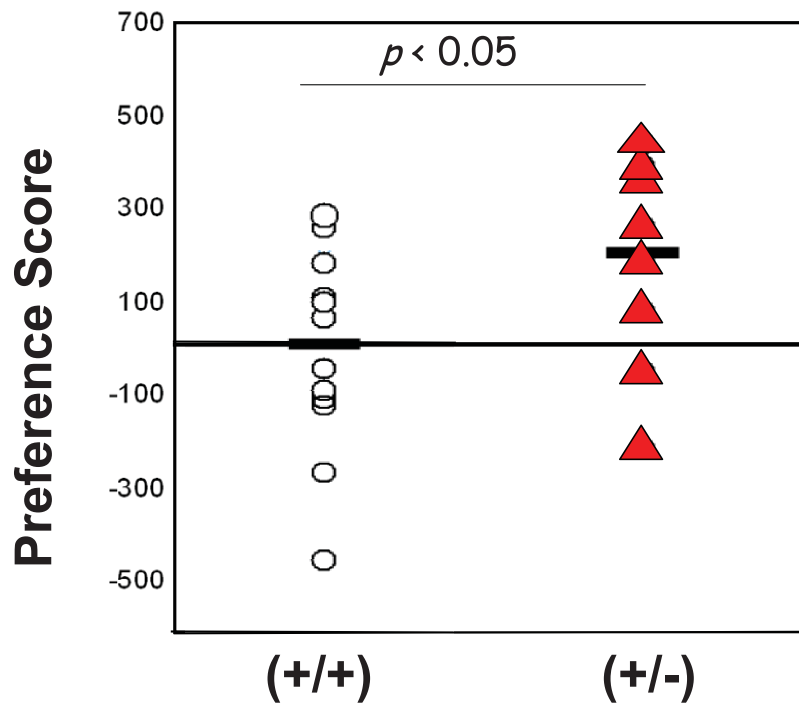


Fig. 6.1. Results of tests for a conditioned place preference (unbiased paradigm) with three pairings of cocaine (20 mg/kg) in Type III *Nrg1* (+/-) versus wild type (+/+) mice (n = 5 / group). Shown is the scatter plot of the preference score (time spent in the drug-paired minus time spent in the saline-paired compartments) plotted on the y-axis as a function of *Nrg1* genotype on the x-axis (+/+) or (+/-) during a cocaine-free place preference test. *Nrg1* (+/-) mice spent significantly ($p<0.05$) more time in the cocaine-paired environment than their wild type age-matched controls. Data were analyzed by using a paired t-test, *$p<0.05$.

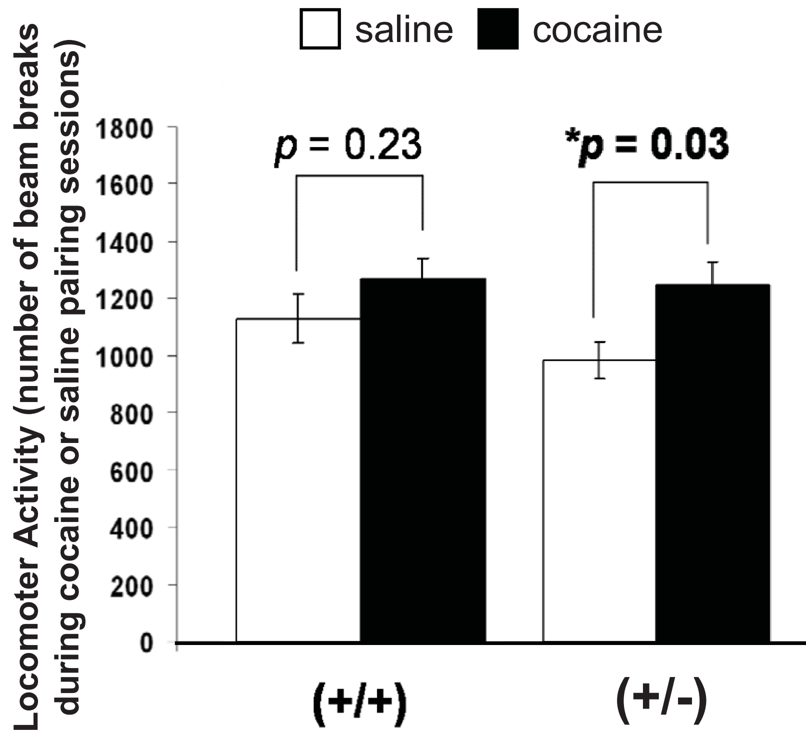
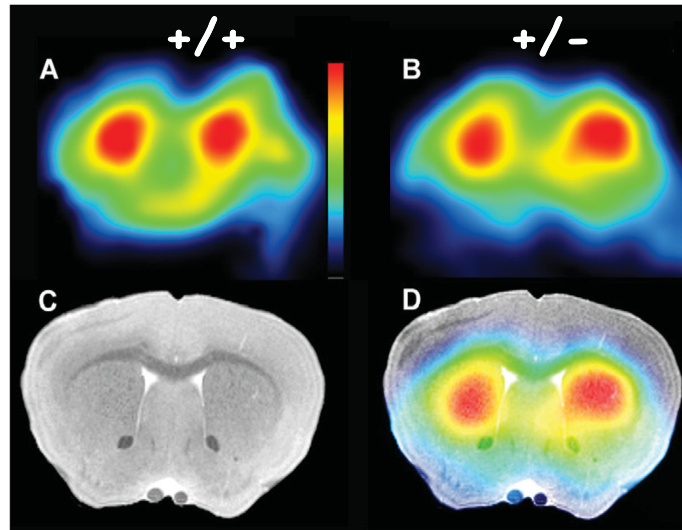


Fig. 6.2 The effects of 20 mg/kg of cocaine on locomotor activity (horizontal movement) in wild type (+/+) and Type III *Nrg1* (+/-) mice (n = 5 / group). The time spent in every compartment during test sessions was automatically recorded by infrared photocells positioned along the walls at the level of animal's head (six on both sides of the two conditioning compartments) during each 20 min conditioning session. The mean number of beam breaks, as an indication of locomotor activity, during 3 paring sessions is graphed in this figure. Paired t-test, $*p<0.05$.



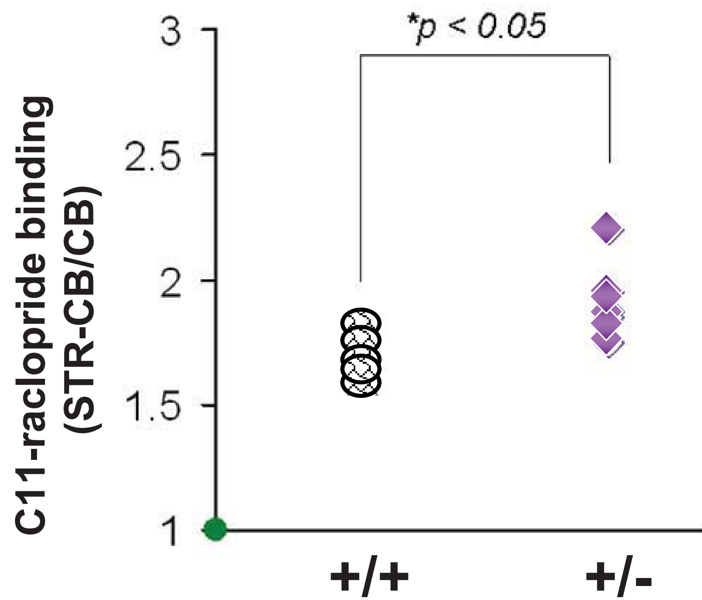


Fig. 6.3. Sample images from positron emission tomography (microPET) with [(11)C]raclopride, dopamine receptor type-2 antagonist, in wild type (+/+) mice (A) and Type III *Nrg1* heterozygous (+/-) mice (B) at the level of the mouse striatum. Shown are the magnetic resonance imaging (C) / microPET overlay (D). The region of interest were drawn on the MRI and transferred to a coregistered microPET image. Shown are the scatter plot of the ^{11}C-raclopride binding ratios, striatum (Str) to the reference region, cerebellum (CB), in (+/+) compared to (+/-) mice (n = 5-6 / group) (D). There was a significant increase ($p<0.05$) in [(11)C]raclopride binding which is indicative of a greater availability of D2 receptors in the (+/-) versus (+/+) mice. (MRI template adapted from Innis et al., 2009)

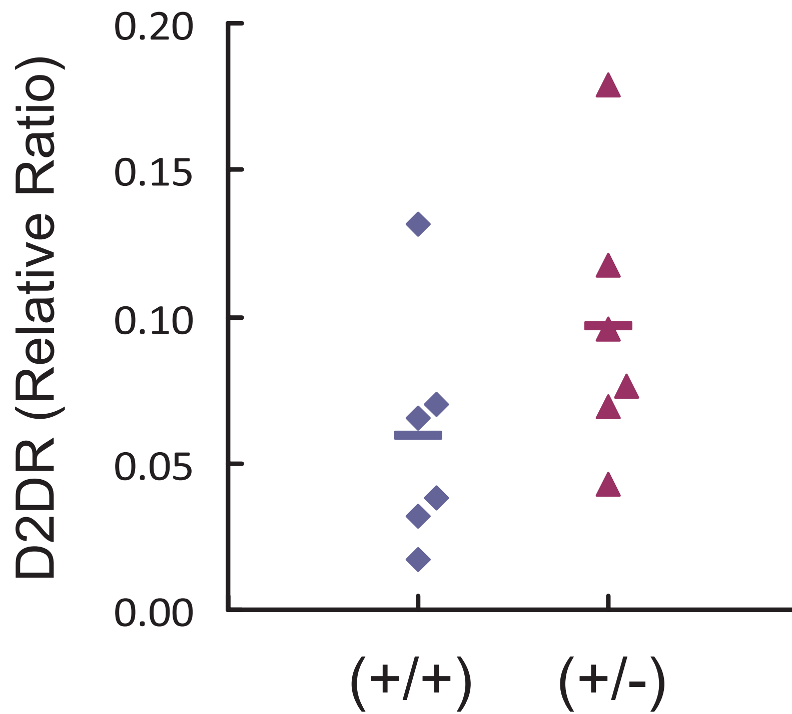


Fig. 6.4. Scatter plot of immunoreactive bands of dopamine D2 receptor protein (D2DR; ~ 98 kDa) normalized to the respective immunoreactive bands of the loading control (GAPDH; ~ 36 kDa) and mean values in striatal tissue in Type III *Nrg1* (+/-) versus wild type (+/+) mice (n = 6 / group). Equal amounts of protein were resolved on a 12 % acrylamide gel, transferred to a nitrocellulose membrane and incubated with a primary antibody for D2DR. Densitometric analysis revealed an increase (64 %; p=0.09) in the band intensity of the Type III *Nrg1* mice.

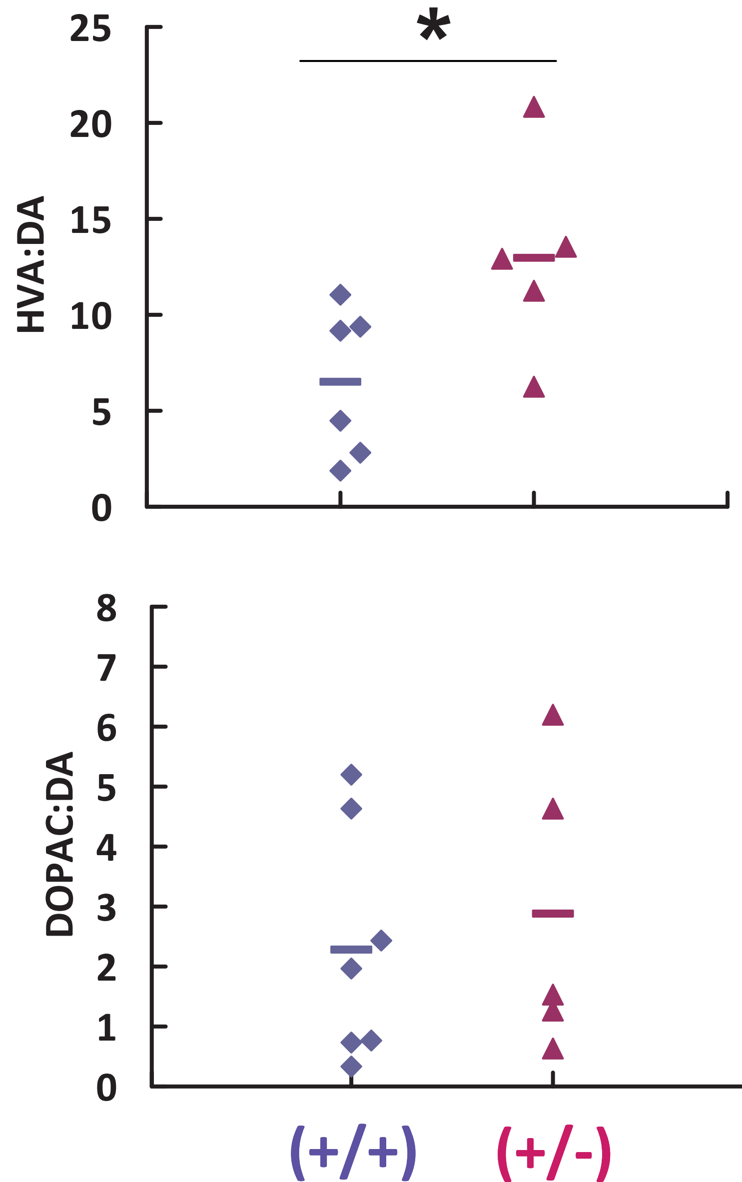


Fig. 6.5. ***In vivo*** **extracellular, homovanillic acid (HVA) / dopamine (A) and 3,4-dihydroxyphenylacetic acid (DOPAC) / dopamine (DA) turnover ratios in the nucleus accumbens of Type III** ***Nrg1*** **heterozygous (+/-) and wild type (+/+) mice(n = 5-7 / group). The basal dialysate fractions were collected over 60 mins after a 90 mins of acclimation of probe placement. The HVA / DA turnover ratios were significantly elevated in the Type III** ***Nrg1*** **mice (p<0.05) versus the wild type group. No change in DOPAC / DA ratios were observed.**

CHAPTER 7

Summary and Conclusion

My thesis work was primarily focused on characterizing the mechanisms by which drugs of abuse, namely solvents, work on the main reward pathway in the brain, the dopaminergic pathway. Since the majority of classical drugs of abuse, like cocaine and amphetamines, directly or indirectly stimulate nucleus accumbens dopamine release (Fowler et al., 1989; Volkow et al., 1990; Paulson and Robbinson, 1996) we tested the hypothesis that the reinforcing effects of toluene (the main component in abused solvents) are mediated, in part, by the dopaminergic system. Therefore, the dopamine-related behavioral effects of toluene were examined using the unbiased conditioned place preference (CPP) paradigm, a model for drug-seeking behavior (Tzschentke, 2007; Shippenberg, 2002). It is worth noting that, the CPP apparatus was modified to deliver controlled amounts of toluene vapors, thus mimicking in animals the direct route of administration used in humans.

The reinforcing effects of toluene inhalation produced an inverted-U shaped dose-response curve (Lee et al., 2006) in a manner typical of psychostimulants that produce their reinforcing effects through the dopamine system. Moreover, a positive correlation was shown between toluene concentration and the animal's locomotor activity, which is again characteristic of CNS stimulants.

In brief, these data give support to the application of an animal model of drug-seeking behavior, conditioned place preference, to study the reinforcing or non-reinforcing properties of less studied solvents, like acetone. It is interesting to note that I

found heterogeneity in the drug-seeking behavioral response even at the reinforcing concentration of inhaled-toluene. Studies have shown that differences in innate tolerance levels do exist which explains the variability in the toluene-induced preference scores. Regardless, altered biochemical signatures between the "high" responding group versus the "low" responding group would help to predict individuals vulnerable to substance abuse. Interestingly, a behaviorally heterogeneous group of Sprague-Dawley rats has been observed using other behavioral paradigms. In fact, human studies have shown that low basal dopamine receptors (D2) in the caudate nucleus can be an indication of addiction vulnerability (Volkow et al., 1990). Further, the validity of this animal model to study drug reinforcement was applied to assess other brain disorders (Geyer, 2008). Clearly, mental illness is intricately tied in with substance abuse. However, despite the high incidence of drug abuse in the schizophrenic population, very few studies are done to understand its comorbidity (Chambers et al., 2001).

At the same time, genetic vulnerability is associated with both diseases. Recently, genome-wide screening for the risk of schizophrenia has led to the identification of susceptibility genes including neuregulin-1 (Stefansson et al., 2009). Transgenic animal models that have targeted genetic mutations related to schizophrenia have given us a unique opportunity to study the impact of the environment and development in mice susceptible to schizophrenia. Importantly, these mice share endophenotypes with schizophrenic patients: impaired prepulse inhibtion, disruption in latent inhibition, social interaction deficits, and many more anatomical and functional abnormalities on the molecular level (Chen et al., 2008; Karl et al., 2007).

I studied the impact of drug abuse on Type III *Nrg1* (+/-) transgenic mice. Interestingly, amphetamine induces a schizophrenia-like state and has been an established animal model to study the positive symptoms of schizophrenia. Thus, we set out to establish a model of comorbid substance abuse and schizophrenia risk using Type III *Nrg1* mutant mice and our established unbiased conditioned place preference experimental design. Cocaine was chosen as our model substance since it is the illicit drug that is most abused by schizophrenics. Our results indicate that Type III *Nrg1* (+/-) mice acquire a place preference to cocaine only after 3 pairings (3 exposures of cocaine; 3 exposures of saline). These present results are consistent with cocaine CPP data in swiss webster mice (Ithak and Martin, 2002), 3 pairings for OF1 (outbred; Oncins France 1) strain mice, and 4 pairings in BKW (University of Bradford bred) mice. Although both biased and unbiased designs were used, the doses are the same across all studies (20 mg/kg). Our results in the wild type mice (C57) indicate that 4 pairings were required to exhibit cocaine-induced CPP.

The number of pairings required to exhibit a drug-induced CPP is not only indicative of the strength of the reinforcing properties of the drug, but also, the drug's ability to produce tolerance which is a physiological response related to dysfunction of the mesolimbic dopamine pathway. Therefore, our results show that a mutation in the *Nrg1* gene may alter dopamine function. The mechanism of this response is unknown at this time, but NRG1 has been shown to modulate the expression of synaptic receptors, like nAChRs (Mathew et al., 2007; Zhong et al., 2008; Portugal and Gould 2008); therefore, similar mechanisms might be involved.

Further, striatal D2 receptors were elevated (*in vivo* and *in vitro*) and as was dopamine metabolism, as measured by extracellular HVA. Altered D2 receptor expression may play a role in the development of behavioral tolerance. Interestingly, increase in D2 receptors is consistent to what has been measured in neuroleptic treated or withdrawal patients (Laruelle, 2000; Wong et al., 1986; Seeman, 1987).

Taken together with the previous reports of endophenotypes shared by Type III *Nrg1* (+/-) mice (Chen et al., 2008; Karl et al., 2007) and schizophrenic patients, the behavioral and neurochemical studies presented in this thesis add to the growing body of literature that show validity of *Nrg1* transgenic mouse model to study schizophrenia and substance abuse comorbidity (Kellendonk et al., 2009; Boucher et al., 2007; Chen et al., 2008; Corfas et al., 2004; Duffy et al., 2008). Here I show two lines of behavioral evidence (CPP and locomotor activity) indicating that Type III *Nrg1* (+/-) mice are more responsive to the psychostimulant effects of cocaine and acquire faster drug tolerance. Further, our neurochemical data indicate increased dopamine metabolism (HVA) along with elevated D2 receptors indicative of a desensitized state often seen in neuroleptic (D2 blockade) treated schizophrenia patients (Smith et al., 1988), suggesting that this population of schizophrenics might be more vulnerable to drug abuse.

In brief, animal modeling has been instrumental in dissecting the pathophysiology of human disease and for the improved design of pharmacotherapies (Geyer, 2008). Granted, not all aspects of the cognitive disease can be faithfully modeled in an animal. This is especially the case in the field of psychology and addiction research (Lipska and Weinberger, 2000). Psychology is aimed at trying to understand human behavior and how the brain works – the nature of emotion, perception and cognitive ability. And so,

whether animals suffer from identifiable mental illness is highly debatable. Nevertheless, clinical psychiatric disorders are primarily a dysfunction of neuronal systems; therefore, when disrupted can affect both human and animal behavior. These models are intended to test potential causative factors and mechanistic hypotheses not to provide a complete equivalent of human disease. A variety of behavioral correlates in animals can serve as approximate models for human brain disorders.

In conclusion, future studies will examine substance abuse and schizophrenia comorbidity in the adolescent population, the age group most vulnerable to drug abuse. Therefore, studies to determine which compounds, like inhaled toluene, can quicken the onset of schizophrenia in the already genetically predisposed population will be of great value. Hence, if we can determine the drugs that exacerbate schizophrenia then drug-induced schizophrenia can be delayed or ceased.

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