Animal Models of Drug Addiction and Autism Spectrum Disorders

DISSERTATION

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Abstract

Autism spectrum disorders are complex and polygenic in nature. Twin studies indicate a role for genetic and environmental factors in the etiology of autism. Environmental influences during prenatal period could adversely affect fetal development. We have attempted to study the effect of different environmental factors on the susceptibility to develop autism. First, we looked at the effect of prenatal exposure to diesel exhaust particles on autism spectrum disorders. We hypothesized that exposure to diesel exhaust particles during pregnancy can induce autism-like behaviors in offspring.

In order to test this, we exposed pregnant mice to high concentration of diesel exhaust particles. We found that mice exposed to diesel exhaust particles during pre and postnatal development showed increased basal locomotor activity. These mice also displayed increased rearing behaviors and elevated levels of repetitive self-grooming in the presence of an unfamiliar mouse. However, no deficits in social interaction, social communication or anxiety-like behavior were found. These results suggest that perinatal exposure to diesel exhaust particles have an impact on mouse development leading to observable changes in mouse behavior, however it may not affect fetal development in a manner that leads to very obvious deficits in social behaviors.

Second, we tested if exposure to food additives during pregnancy induced autism-like behavior in the offspring. We fed pregnant mice with different kinds of food additives and tested the offspring for autism-like behavior. We report that a subset of
mice exhibit reduced social interaction following additive treatment, although there were other groups of additives that had no effect on mouse social behaviors. These findings are preliminary and studies are currently on-going to consolidate these findings.

Third, we generated a knockin mouse of protein tyrosine phosphatase protein (PTPRT). PTPRT is a transmembrane receptor protein that is expressed in high levels in the brain and spinal cord. It is known to interact with proteins belonging to neuroligin and neurexin family and a recent study found PTPRT to be a candidate gene for autism susceptibility. We generated a knockin mouse carrying a mutated PTPRT (D1046A) with an inactivated phosphatase domain. We then conducted behavioral analyses of these mice to determine if inactivation of phosphatase function of PTPRT has an impact on mouse behaviors relevant to behavioral deficits seen in ASD patients. We found that inactivating the phosphatase function of PTPRT significantly increased social interaction in mice indicating that PTPRT/RPTPp may play a role in shaping neural pathways important for social behaviors.

And finally, we delineate functions of dopamine D1 and D5 receptors expressed within the nucleus accumbens in mediating cocaine’s effects. Cocaine inhibits dopamine, serotonin and norepinephrine transporters. Dopamine signaling is mediated by dopamine D1-like (D1R and D5R) and D2-like family of receptors. There is no clear separation of function between D1R and D5R in mediating cocaine’s effects, due to lack of pharmacological ligands that distinguish the two. We used short hairpin RNA’s to selectively downregulate these receptors in nucleus accumbens and assessed their role in mediating cocaine’s effects. We found that D1R in the nucleus accumbens may be critical
for mediating cocaine’s acute locomotor effects as well as cocaine induced locomotor sensitization. D5R, on the other hand may have an inhibitory role in mediating cocaine induced locomotor behavior at low doses.
Dedication

To

Appa, Amma, and Janani
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Abbreviations

D1R; Dopamine D1 receptor
D5R; Dopamine D5 receptor
NET; Norepinephrine transporter
DAT; Dopamine transporter
SERT; serotonin transporter
shRNA; short hairpin RNA

AAVshGFP – shRNA against green fluorescent protein
AAVD1shR; shRNA against dopamine D1 receptor
AAVD1shR; shRNA against dopamine D1 receptor

NAc; nucleus accumbens
PFC; prefrontal cortex

qRT-PCR; quantitative real-time polymerase chain reaction
VTA; ventral tegmental area
SN; substantia nigra
ASD; autism spectrum disorders
DEP; diesel exhaust particles

PTPRT; protein tyrosine phosphatase receptor type T
Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by impaired social interaction, deficits in social communication and repetitive and restrictive behaviors. According to the Center for Disease Control (CDC), 1 in 88 children are diagnosed with autism spectrum disorders in the United States (CDC, 2012). The condition is predominant in males than females by a ratio of 4:1. According to the latest diagnostic and statistical manual, ASD is categorized into 4 disorders: autistic disorders, Asperger's disorder, childhood disintegrative disorder and pervasive developmental disorder not otherwise specified (DSM, V).

The genetic basis for ASD etiology has been well established. Some of the earliest studies found concordance rates for broader definition of ASD to be close to 90%, whereas 60% concordance rates were found in monozygotic twins for autism alone (Etherton et al., 2009, Calderon-Garciduenas et al., 2011, Peca et al., 2011). With stricter evaluation techniques that separated autism from other developmental disorders such as mental retardation, studies indicate the influence of genetic factors contributing to the etiology of ASD may have been overestimated. This suggests that environmental factors may play a much larger role than previously thought (Hallmayer et al., 2011). Given the heterogeneity in ASD etiology, it is likely that a combined effect of genetic and
environmental factors may contribute to ASD incidences. Some of the proposed environmental factors contributing to ASD etiology are exposure to chemicals, changes in perinatal environment, maternal diet and parental age of conception. There is strong evidence linking parental age of conception and the likelihood of offspring developing ASD (Block et al., 2012). One of the first studies linking chemical exposure to ASD was associated with use of thalidomide by pregnant women. A medication prescribed to treat morning sickness in pregnant women; it caused severe birth defects in the offspring. Small subsets of children were also diagnosed with autism (Stromland et al., 1994).

*In utero* exposure to valproic acid, an anticonvulsant drug, has also been suggested as a risk factor for autism spectrum disorders (Christensen et al., 2013). The nervous system of a developing fetus is vulnerable to environmental insults. Critical events during fetal development such as neurogenesis, migration, synapse formation are greatly influenced by exposure to environmental agents (Monif et al., 1972, Gordeeva et al., 1990, Shi et al., 2005). We have discussed the effects of two environmental factors; diesel exhaust particles (DEP) and food additives. DEP is a component of air pollution. A recent study proposed that children living near highways have a greater risk of developing autism (Volk et al., 2011). Exposure to diesel exhaust particles during pregnancy is known to induce adverse effects including a decrease in sperm count and an increase in serum testosterone levels (Yoshida et al., 2006). Within the nervous system, exposure to DEP is known to decrease dopamine turnover in the striatum and impair basal locomotor activity (Yokota et al., 2009). We hypothesized that exposure to DEP during pregnancy would
induce autism-like phenotype in the offspring. A brief introduction along with results are presented and discussed in chapter 2. Another potential environmental factor that could be involved in autism etiology is food additives. Naturally occurring food additives have been used since 300 BC. In the United States, the first synthetic dye was discovered in 1856. The Food and Drug Administration (FDA) began to regulate use of food additives in 1880s. Food additives are divided into numerous categories, most prominent ones are; preservatives, food colors and artificial sweeteners. Over the four past decades, there has been a 10 fold increase consumption of food additives. We fed a diet of different food additives to pregnant mice and screened offspring for deficits in social interaction using the 3-chambered social interaction task. Results from screening four groups of food additives are presented in chapter 3.

In the third chapter we describe a genetic mouse model for Autism spectrum disorders. In the last few years numerous mouse models of proteins associated with ASD have been reported. Genes involved in its etiology can be classified into the following types, syndromic genes, ASD-associated genes and genes with marginal evidence for ASD association. Syndromic genes are those that have been previously been associated with other syndromes but exhibit ASD-like phenotype. Genes such as MECP2, FMR1, SHANK3 and PTEN belong to this category. Mice with mutation in FMR1 gene are born with mental retardation and seizures besides exhibiting symptoms of autism (Hagerman et al., 1986). Mutant FMR1 knockout mice show severe deficits in social interaction and spatial learning (Mineur et al., 2002, Mineur et al., 2006). Methyl-CpG binding protein-2
(Mecp2) is a transcription regulator that is involved in chromatin remodeling. In humans, mutation in Mecp2 causes Rett’s syndrome. This condition is characterized by severe deficits in cognitive and motor function. Patients diagnosed with Rett’s syndrome also show ASD-like phenotype as the condition progresses with age (Chahrour and Zoghbi, 2007). Phosphatase and Tensin homolog of chromosome 10 (PTEN) is a phosphatase that involved in the dephosphorylation of phosphatidylinositol-(3,4,5)-triphosphate (PIP3). It is a tumor suppressor gene that negatively regulates the Akt pathway (Song et al., 2012). Individuals with mutations in the PTEN gene display aberrant social behavior and macrocephaly (Waite and Eng, 2002).

SHANK3, on the other hand, belongs to a family of scaffolding proteins and is involved in the formation of the post-synaptic density complexes. Mutation in SHANK3 results in mental retardation. Mutation in SHANK3 has also been found in a small population of autistic patients (Gauthier et al., 2009). SHANK3 mutant mice also display impairments in social interaction and excessive self-grooming (Peca et al., 2011). Contactin-4 (CNTN4) and neurexins (NRXN) are two prominent genes that belong to the second category; strong ASD associated genes. These genes have been identified following extensive genome-wide association studies. Neurexins are presynaptic cell adhesion proteins that are known to interact with neuroligins, which are expressed postsynaptically (Sudhof, 2008). Disruptions in Neurexin-1 have been associated with ASD phenotype (Kim et al., 2008). Although social behaviors were normal in neurexin-1 knockout mice, they showed impaired grooming behaviors (Etherton et al., 2009).
And genes such as oxytoxin (OXT), neureligins (NLGN), and reelin (RLN) belong to a class of genes whose ASD association is based on minimal or suggestive evidence. In chapter 2, we present data from behavioral characterization of protein tyrosine phosphatase receptor type T (PTPRT), which can be classified in the third class of genes along with oxytocin, neureligins and reelin. It is a transmembrane protein expressed highly in the brain and is known to interact with synaptic proteins such as neureligins. A recent analysis of a large autism pedigree identified PTPRT as a candidate gene for autism susceptibility (Allen-Brady et al., 2009). A description of the mouse and results from behavioral experiments are presented in chapter 4.
Chapter 2: Prenatal and early life exposure to high level diesel exhaust particles leads to increased locomotor activity and repetitive behaviors in mice

2.1 Abstract

Abundant evidence indicates that both genetic and environmental factors contribute to the etiology of Autism Spectrum Disorders (ASDs). However, limited knowledge is available concerning these contributing factors. An epidemiology study reported a link between increased incidence of Autism and living closely to major highways, suggesting a possible role for pollutants from highway traffic. We investigated whether maternal exposure to diesel exhaust particles (DEP) negatively affects fetal development leading to autism-like phenotype in mice. Female mice and their offspring were exposed to DEP during pregnancy and nursing. Adult male offspring were then tested for behaviors reflecting the typical symptoms of ASD patients.

Compared to control mice, DEP-exposed offspring exhibited higher locomotor activity, elevated levels of self-grooming in the presence of an unfamiliar mouse and increased rearing behaviors, which may be relevant to the restricted and repetitive behaviors seen in ASD patients. However, the DEP-exposed mice did not exhibit deficits in social interactions or social communication which are the key features of ASD. These results suggest that early life exposure to DEP could have an impact on mouse
development leading to observable changes in animal behaviors. Further studies are needed to reveal other environmental insults and genetic factors that would lead to animal models expressing key phenotypes of the autism spectrum disorders.

2.2 Background and Introduction

Autism Spectrum Disorders (ASDs) are neurodevelopmental disorders characterized by deficits in sociability, impaired social communication, restricted and repetitive behaviors (American Psychiatric Association, 2013). Evidence from twin studies has indicated the contribution of genetic and environmental factors to ASD, as shown by almost 90% concordance rates in monozygotic twins (Ronald et al., 2006, Taniai et al., 2008, Lichtenstein et al., 2010, Hallmayer et al., 2011, Peca et al., 2011).

Although genetic and linkage studies have identified many candidate genes, the exact genetic causes have not been determined for a large majority of autism cases. A number of epidemiological studies indicate gene-environment interaction as possible contributors in ASD (Hultman et al., 2002, Newschaffer et al., 2007, Hallmayer et al., 2011). Events during pre- and post-natal stages can adversely affect normal development of the fetus (Perera and Herbstman, 2011). Prenatal stress combined with a reduced serotonin transporter expression has been shown to produce deficits in social interaction, an important feature of autism (Jones et al., 2010). Similarly, insults during early pregnancy via exposure to chemical and biological agents have been linked to autism etiology in a subset of cases (Chess et al., 1978, Williams et al., 2001). Diesel exhaust
particle (DEP), a component of air pollution, is one such environmental factor that has increasingly been associated with negative health outcomes (Sydbom et al., 2001, Silverman et al., 2012). Prenatal exposure to DEP may produce abnormalities in reproductive functions and changes in immune responses (Watanabe and Ohsawa, 2002, Yoshida et al., 2006, Niedzwiecki et al., 2012). Exposure to DEP during pregnancy can also cause an increased frequency of fetal DNA deletions (Reliene et al., 2005). High concentration (1000 µg/m³) exposure to DEP in utero results in elevated levels of serum testosterone and also causes a decrease in sperm production (Yoshida et al., 2006). A similar level of exposure during prenatal period has also been shown to produce a significant decrease in dopamine turnover within the striatum and reduce locomotor activity in mice (Yokota et al., 2009).

A recent epidemiological study also found an association between residential proximity to freeways, a proxy for traffic related pollutants and incidences of autism (Bragiel et al., 1965). The aim of this study is to test the hypothesis that high concentration exposure of DEP to mice during gestation and nursing could adversely impair fetal brain development leading to behaviors relevant to autism spectrum disorders.

2.3 Materials and Methods

Animals. Eight-week-old B6C3F1 male and female mice (Jackson laboratories; Bar Harbor, ME) were allowed to acclimate for one week at the New York University
(NYU) animal facility. Two 9-week-old females were then paired with a single male for up to four days and checked for the presence of vaginal plugs to confirm mating. The successfully mated female mice were exposed to DEP as described below. The DEP exposure continued for both the dams and pups during nursing and ended 1 week after birth. All procedures conducted at NYU were approved by the NYU School of Medicine Institutional Animal Care and Use Committee (IACUC). The weaned male and female offspring were transferred from NYU to the Ohio State University (OSU). Animals were maintained on a 12 hour dark/light cycle with ad libitum access to water and food. At the age of 6 weeks, behavioral experiments were performed on both offspring sexes. All animal procedures were approved by The Ohio State University Internal laboratory Animals Use Committee.

Diesel exhaust exposure Diesel exhaust was produced by a 5500-watt single cylinder diesel engine generator (Yanmar YDG 5500EE-6EI; Osaka, Japan) that contains a 418-cc displacement engine (Model LE100EE-DEGY6) as previously described (Lin et al., 2010). The engine was operated using an ultra-low-sulfur diesel fuel and the diesel exhaust particles (DEP) were diluted to a desirable level through a serial dilution system with HEPA-filtered ambient air, and routed to a 1 m$^3$ flow-through exposure chamber. Mice were exposed at a concentration of 1000 µg/m$^3$ or filtered air for 4 hours/day, (5 days/week) from the beginning of gestation until the first week after birth. Since mice are likely to be less sensitive to pollutants than humans, we and other investigators choose the DEP concentration of 1000 µg/m$^3$. This concentration is much higher than human
exposures to particulate matter from diesel exhaust which is approximately 50ug/m$^3$ in major US cities (National Ambient Air Quality Standards).

**Behavioral Assay**

*Open Field locomotion* Spontaneous locomotor activity was measured in a 40 cm x 40 cm apparatus placed in a sound-attenuating chamber for 20 minutes (Fonken et al., 2011, Brielmaier et al., 2012). Animals were placed in the center of the apparatus at the beginning of the test. Total distance travelled and time spent in the center (20 cm x 20 cm) of the box was measured using tracking software (ANYmaze, Stoelting Co.) Grooming and rearing behaviors were also scored in the same paradigm by an observer blind to treatment groups.

*Elevated plus maze* The elevated plus maze consists of two open arms (35x6 cm$^2$) and two closed arms (35x6x22 cm$^3$) at right angles to each other. Mice were placed at the intersection of the two arms and allowed to explore the maze for a total of 5 minutes (Holmes et al., 2003). Time spent in the open versus closed arms and the number of entries into the arm was measured using tracking software. Arm entry was defined as the placement of all fours limbs in the arm.

*Social approach Task* Social approach was measured in a 3-chambered apparatus using a previously validated procedure (Crawley, 2007). The test is divided into three 10 min stages. In the first 10 min stage, mice were allowed to freely explore the apparatus containing novel wire cages in the side chambers. This is followed by another 10 min test; wherein an unfamiliar mouse (stranger 1) is enclosed under the wire cage in one of
the side chambers. The third phase involves the introduction of another unfamiliar mouse (stranger 2) which is placed under the wire cage that was previously empty. Time spent in the chambers during all three stages was measured. Cylindrical wire cages (Galaxy Cup, Spectrum Diversified Designs, Streetsboro, OH) were used to hold the stranger mice. The location of the stranger mice and wire cage was counterbalanced between test animals. Age matched C57BL/6j mice were used as unfamiliar stranger mice and were habituated to the wire cages for 15 minutes/day for 3 days prior to the test.

*Reciprocal social interaction* Reciprocal social interaction was recorded for 30 minutes in an apparatus measuring 30 x 15.5 cm. Two animals from the same treatment group (but different home cages) were placed in apparatus containing fresh bedding (McFarlane et al., 2008, Peca et al., 2011). The following behaviors were scored for 15 minutes and quantified by a person blind to the treatment groups. These behaviors included: nose to nose sniffing, nose to anogenital sniffing, and fighting behavior.

*Grooming and rearing behaviors* Grooming and rearing behaviors of each of the 2 mice were also scored from video recordings of reciprocal social interactions described above. Time spent self-grooming and number of grooming episodes was scored (Pearson et al., 2011). A grooming bout is defined as a single episode of uninterrupted sequence of body grooming. Similarly time spent rearing and number of rearing episodes were recorded and scored. A rearing episode is defined as number of times the mouse stands upright on its hind limbs. Both behaviors were scored and quantified by an experimenter blind to the treatment groups.
Social transmission of food preference was performed using a method published previously (Kogan et al., 1997, Rampon et al., 2000, Wrenn et al., 2003, McFarlane et al., 2008). Animals were randomly divided into two cohorts within each treatment group and designated as either demonstrators or observers. Demonstrate mice were habituated to flavored food (1% Cinnamon or 2% Cocoa) mixed with powdered mouse chow in a jar (Dyets Inc., PA) 2 days prior to the experiment. Mice designated as demonstrators were then food deprived for 18 hours. The following day, half the animals in the demonstrator group received 1% cinnamon (Kroger, Cincinnati, OH) and the rest received 2% cocoa (Hershey’s cocoa, Hershey, PA) in their home cage for an hour. Amount of food consumed was noted and mice that failed to consume more than 0.1g were removed from the study. This phase was followed immediately by a one-on-one interaction with observer mice from the same treatment group for 30 min. The demonstrator and observer mice in each treatment group were socially naïve and from different cages. Mice from both cohorts were returned to their respective cages after the 30 minute interaction session. At this stage, food from the observer animals was removed. Twenty-four hours later, observer mice were given a choice between cocoa and cinnamon flavored food. The jars were then weighed and the amount of food consumed was noted.

Statistical Analyses Data analysis was performed using SPSS software (version 19). Repeated measures ANOVA were used to compare locomotion across time between
the two treatment groups (Control vs. DEP) in the open field and time spent in chambers (Stranger vs. Empty, Stranger 1 vs. Stranger 2) in the social approach task. One way ANOVA was used to analyze time spent in the open arm and number of entries in the elevated plus maze, grooming and rearing behaviors and percent cued food consumed in the social transmission of food preference task.

2.5 Results

DEP exposure

To investigate the effect of DEP exposure during development, animals were exposed to DEP during pregnancy and nursing. The DEP treatment did not affect litter sizes which averaged ~9 pups per litter. The body weights of animals in the two treatment groups were also similar (data not shown) and no obvious defects in the physical health of animals were observed as a result of treatment. The weaned mice were then shipped to OSU and were allowed to habituate to the new environment for a week. All animals were tested in the following order, open field locomotion at age 5-6 weeks; social behavior tests at age 6 weeks; reciprocal social interaction, self-grooming, and rearing behaviors at week 7; social transmission of food preference was tested at week 8 and elevated plus maze at week 9.
Open field locomotor activity

Open field locomotor activity was measured in a locomotor box for 20 minutes. Fig. 2.1 shows the distance travelled in an open field apparatus over time (Fig. 2.1A), total distance travelled (Fig. 1B) and the % time spent in the center of the chamber (Fig. 2.1C) by control and DEP treated mice. Repeated measures ANOVA reveals a significant difference in locomotor activity in the first 5 minutes of testing between the DEP treated group (n=24) and the controls (n=24) (Fig 1A, \( F_{1, 46} = 10.919, p=0.002 \)). One way ANOVA showed a significant main effect of treatment on overall locomotor activity (distances travelled in 20 min) (Fig. 2.1B, \( F_{1, 46}= 5.997, p=0.018 \)). No significant differences was observed in the amount of time spent in the center area of the open field apparatus between control and DEP treated animals (Fig. 2.1C, \( F_{1, 46}= 0.723, p=0.400 \)).

Elevated plus maze

Fig 2.2 shows the time spent in the open arms (Fig. 2.2A) and the total number of entries into the open arms (Fig. 2.2B) of the elevated plus maze by control and DEP treated groups. DEP treated animals (n=24) did not differ from the control group (n=24) in the amount of time spent in the open arms (Fig. 2.2A, \( F_{1, 45} = 0.153, p=0.697 \)). Similarly, the number of entries made into the open arm also did not differ significantly across treatment groups (Fig. 2.2B, \( F_{1, 45} = 0.318, p=0.576 \)).
Social Approach Tests

Social approach was tested using a three chambered apparatus and the test mice did not show preference for either of the side chambers (data not shown). Fig. 2.3A shows that the DEP treated mice exhibited significant preference (n=24, *p<0.05, One way anova) for the unfamiliar mice (stranger 1) over the empty wire cage just like controls (n=24, *p<0.05). There was no significant main effect of treatment (DEP exposure) on the time spent in the chamber containing stranger 1 over the chamber containing an empty wire cage as measured by repeated measures ANOVA (F\(_{1,46}\) = 1.231, p=0.273). In the third 10-min phase of the test, a new stranger mouse (stranger 2) was placed in the side chamber under the previously empty wire cage. The test mouse was then monitored and time spent with the now familiar mouse (Stranger 1) and unfamiliar mouse (Stranger 2) were recorded and shown in Fig. 2.3B. Both groups of mice showed preference for the new unfamiliar mice but there was no significant difference between the DEP treated and control groups (repeated measures ANOVA, (F\(_{1,46}\) = 0.129, p=0.721).

Reciprocal Social Interaction

Fig 2.4 shows the number of episodes of nose to nose sniffing (Fig. 2.4A) and nose to anogenital sniffing (Fig. 2.4B) between pairs of control mice and pairs of DEP treated mice. There appears to be a reduced frequency of nose to nose and nose to anogenital sniffs between DEP treated mice (8 pairs, n=8) compared to the control group
(n=12). However, the differences are not statistically significant for the nose to nose sniffing (Fig. 2.4A, $F_{1,18} = 0.925$, $p=0.349$) or nose to anogenital episodes (Fig. 2.4B, $F_{1,18} = 1.921$, $p=0.183$).

**Social Transmission of Food Preference**

Two food flavors were tested and no preference for either flavor was observed for both treatment groups (data not shown). Fig. 2.4C shows that the observer mice from both groups consumed substantially more food mixed with a flavor (cued food) that the demonstrator mice consumed, over a non-cued food suggesting effective “communication” between mice. Levene’s test revealed unequal variances between the two groups ($p=0.004$) hence data was analyzed using one way ANOVA with Welch correction. However, there was no significant difference in the percentages of cued food consumed between the control (n=11) and DEP (n=12) treated mice ($F_{1, 11.125} = 3.27$, $p=0.098$).

**Self-Grooming and Rearing Behavior**

Self-grooming and rearing behaviors were scored during the reciprocal social interaction paradigm when two animals where placed together in the apparatus. Fig. 2.5 shows time spent self-grooming (Fig.2.5A) and number of rearing episodes (Fig.2.5B) in 15 minutes. Data are presented as mean ± SEM. DEP treated animals (n=16) spent significantly longer time self-grooming compared to controls (n=24) (Fig. 2.5A, $F_{1,18} = 8.282$, $p=0.010$). There was also a significant increase in the number of rearing episodes.
in the DEP treated animals compared to control animals (Fig. 2.5B, $F_{1,18} = 4.698$, $p=0.04$). Additionally, self-grooming and rearing behaviors were also scored during the open field locomotor activity task when a single mouse was in the apparatus. Fig. 2.5C shows time spent grooming and Fig. 2.5D shows rearing episodes in a 15 minute session. There was no significant difference in time spent self-grooming in DEP treated mice (n=24) compared to controls (n=24) (Fig. 2.5C, $F_{1,46} = 0.084$, $p=0.774$). However, DEP treated mice displayed significantly higher number of rearing episodes compared to controls ($F_{1,46} = 4.527$, $p=0.039$).

2.6 Discussion

In the current study, we have investigated the possibility that exposures to diesel exhaust particles during gestation and early life may impair brain development leading to autism-like symptoms. We report that mice exposed to diesel exhaust particles during pre and postnatal development exhibit an increase in locomotor activity and rearing behaviors. These mice also display elevated levels of repetitive self-grooming only in the presence of an unfamiliar mouse. However, these animals did not exhibit deficits in social interaction or anxiety-like behavior and display normal social communication. These results suggest that exposure to diesel exhaust particles during early development may not have a very strong impact on mouse development leading to very obvious deficits in social behaviors. However, significant increases in stereotyped behaviors were observed which may be relevant to the repetitive/compulsive behaviors seen in autistic
patients. These findings support the hypothesis that, besides genetic factors, pre- and post-natal environment can contribute to behaviors similar to those observed in an autistic phenotype.

Animal studies have shown that exposure to DEP, a common component of air pollution, particularly in urban environments, can alter endocrine function and cause an increase in pro-inflammatory cytokines within the central nervous system (Levesque et al., 2011). In utero exposure of DEP has been shown to produce deficits in learning and memory (Hougaard et al., 2008). However, no study has reported the effect of DEP on social behaviors. A recent epidemiological study reported a correlation between autistic incidences and residential proximity to freeways considered as a proxy for traffic related pollutants, suggesting that environmental pollutants could be possible risk factors for autism-like behavior (Bragiel et al., 1965).

Since there are no biochemical markers to test for autism, we investigated animal behaviors that have face validity to some of the core symptoms of autism. We tested our animals in a variety of behavioral paradigms that measures sociability, social communication and stereotyped behavior. Our results indicate that animals treated with DEP spend more time with an unfamiliar mouse than a novel object, similar to the control mice, indicating that prenatal exposure to DEP does not alter social behaviors in mice. Besides measuring the animal’s sociability in a 3-chambered apparatus, we also recorded parameters such as nose-nose sniffing, and nose to anogenital sniffing. Although the data shows a trend of decrease in these behaviors in the DEP treated animals compared to the
controls, the difference failed to reach statistical significance. Social transmission of food preference task (STFP) was used to assess social communication in these animals. The test measures animal’s ability to use social cues acquired via interaction with another mouse to then make a choice. The test has been reported to have potential face validity to certain features of autism (Crawley, 2007). However, significant differences between control and DEP exposed animals on the STFP task was not observed here. Taken together, these findings suggest that prenatal and early life exposure to DEP do not have adverse impact on mouse development that results in a significant deficit in social behavior or communication.

Repetitive and restrictive behaviors are one of the diagnostic criteria for autism spectrum disorders (American Psychiatric Association, 2000). Mouse models of autism have previously been shown to display excessive self-grooming, rearing and other stereotyped behaviors (Etherton et al., 2009, Blundell et al., 2010, Chao et al., 2010, Peca et al., 2011). A dysfunctional fronto-striatal circuitry has been suggested to contribute to restrictive behavior (Langen et al., 2012). Increased striatal volume has also been positively correlated with repetitive behavior in autistic patients (Hollander et al., 2005). In utero exposure to low concentrations of DEP also results in enhanced dopamine concentration within the pre frontal cortex (PFC) and reduced dopamine turnover in the striatum and nucleus accumbens (Suzuki et al., 2010). We found that DEP exposed mice when compared to controls, exhibited elevated levels of self-grooming only in the presence of an unfamiliar mouse. Interestingly, there was no difference between DEP
treated mice and controls in time spent self-grooming when the mice were alone. The increased self-grooming in DEP treated mice might be due to anxiety induced by the presence of an unfamiliar mouse. These results are similar to previous reports that show higher grooming times during social interaction in a mouse model of fragile X syndrome (King-Herbert et al., 2010, Pearson et al., 2011). Previous studies have also shown that in small population of children diagnosed with autism, higher anxiety levels are associated with a greater frequency of repetitive behaviors (Brodkin, 2008, Mines et al., 2010, Dewan et al., 2011). However, mice may groom and rear more for a variety of reasons which may or may not be related to autism (van Erp et al., 1994, Button et al., 2012). Additional experiments such as reversal learning and marble burying are needed to further characterize how perinatal DEP exposures affect repetitive behaviors and restricted interests.

A subset of children diagnosed with autism spectrum disorders display a hyperactive phenotype (Sinzig et al., 2009). Hyperactivity has also been reported in a few mouse models of autism (Penagarikano et al., 2011). In our study, DEP exposed animals display an increased locomotor activity in an open field environment compared to controls, suggesting some effect of early life DEP exposure on the development of brain structures underlying motor behavior. Another recent study also showed that prenatal DEP exposure has an impact on locomotor activities in mice (Suzuki et al., 2010). However, this study reported a decrease in locomotor activity as a consequence of DEP exposure. There are a few differences between the two studies. First, the strains of mice
used in the two studies are different, we used an inbred strain B6C3F1, whereas they used
ICR mice, an outbreed strain. Second, the durations of DEP exposure in the two studies
are different. We exposed pregnant dams during pregnancy and nursing for 4h/d, 5d/wk
whereas the other group exposed the mice for 8h/d, 5d/wk from day 2 through Day 17 of
gestation. These disparities could have contributed to the observed differences between
the two studies. B6C3F1 mice are thought to be more sensitive to toxic materials and thus
are commonly used in toxicological studies (Baur et al., 2011). There are no obvious
abnormality reported for this strain in its baseline behaviors, health, and physiology. One
study reports that B6C3F1 appeared to display a more anxious phenotype compared to
C57 mice (Benatti et al., 2011). However, we did not observe any obvious anxiety like
phenotype.

A small proportion of patients diagnosed with ASD present with symptoms that
are commonly associated with psychological disorders like ADHD and anxiety (Muris et
al., 1998, Esbensen et al., 2003, Goldstein and Schwebach, 2004, Brereton et al., 2006,
Leyfer et al., 2006, Tillfors et al., 2009). Some of the anxiety symptoms manifest as
simple phobias, separation anxiety disorders or agoraphobia (Muris et al., 1998). In order
to test for anxiety-like behavior we performed elevated plus maze and open field test. We
observed no significant differences between the two groups of mice in the amount of time
spent in the center of the open field, a measure of anxiety-like behavior in animals, which
is often used to assess anxiety. This was supported by no differences observed in the time
spent in the open arm or the number of transitions into the open arm of an elevated plus

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maze. Although we do not understand molecular mechanisms underlying these behavioral deficits, changes in immune function following DEP exposure have been reported by many studies. Exposure to DEP is known to increase protein levels of TNFα and ILα in the striatum of rats (Gerlofs-Nijland et al., 2010). Imaging studies have identified increased levels of activated microglia in specific brain regions of children diagnosed with autism (Suzuki et al., 2013). Microglia have been shown to regulate synaptic pruning through a complement dependent signaling mechanism (Schafer et al., 2012). It is possible that prolonged exposure during pregnancy may have altered microglia function leading to impaired synapse development.

Taken together, our results suggest that early life exposure to DEP could have an impact on mouse development leading to observable changes in animal behaviors. It is likely that multiple environmental insults in combination of genetic factors would have stronger impact and result in animals with other key phenotypes of the autism spectrum disorders.
Figure 2.1 Open field locomotor activity. (A) Distance travelled over time. Repeated measures ANOVA reveals a significant difference in locomotor activity in the first 5 minutes of testing between the DEP treated group (n=24) and the controls (n=24) ($F_{1,46} = 10.919, * p=0.002$). (B) Total distance travelled in 20 minutes. One way ANOVA showed a significant main effect of treatment on locomotor activity ($F_{1,46} = 5.997, * p=0.018$). (C) No significant difference was observed in the amount of time spent in the center area of the open field apparatus between control and DEP treated animals ($F_{1,46} = 0.723, p=0.400$). Data are presented as mean ± SEM.
Figure 2.2 Elevated plus maze. (A) Time spent in the open arm in a 5 min test. DEP treated animals (n=24) did not differ from the control group (n=24) in the amount of time spent in the open arm ($F_{1,45} = 0.153, \text{NS}$). (B) Number of entries into the open arm. No significant difference was observed in the number of entries into the open arm between treatment groups ($F_{1,45} = 0.318, \text{NS}$). Data are presented as mean ± SEM.
Figure 2.3 Social approach task. (A) Time the test mice spent in the center chamber, the side chamber containing an unfamiliar mouse (stranger 1) enclosed in a wire cage or the side chamber having an empty wire cage. DEP treated animals (n=24, *p<0.05) displayed normal sociability, similar to their controls (n=24, *p<0.05). (B) Time spent in each of the 3 chambers when a new unfamiliar mouse (stranger 2) was placed under a previously empty wire cage. DEP treated animals showed similar preference for social novelty as their control counterparts.
Figure 2.4 Reciprocal social interaction. Two mice from the same treatment groups but from different home cages were placed in a novel environment and number of nose-to-nose sniffing episodes (A) and number of nose to anogenital sniffing episodes (B) were recorded. No significant differences were observed between control (n=12) and DEP treated (n=8) animals on number of episodes of nose to nose sniffing ($F_{1,18} = 0.925, p=0.349$) or nose to anogenital sniffing ($F_{1,18} = 1.921, p=0.183$). (C) Social transmission of food preference. No significant differences were observed between the control (n=11) and DEP treated animals (n=12) in the cued food consumed ($F_{1,11.125} = 3.27, NS$). Data are presented as mean ± SEM.
Figure 2.5 Self-Grooming and rearing behavior. (A and B) Two mice were placed in a novel environment and grooming and rearing behaviors of each animal were scored. (A) DEP (n=16) treated animals spent significantly longer time self-grooming compared to controls (n=24), $F_{1,18} = 8.282, *p=0.010$) (B) Number of rearing episodes. There was a significant increase in number of rearing episodes in the DEP treated animals compared to control animals, $(F_{1,18} = 4.698, *p=0.04)$. (C and D) Single mouse was placed in test apparatus and self-grooming and rearing behaviors were scored. (C) No significant differences were observed between control (n=24) and DEP treated mice (n=24) in the amount of time spent self-grooming $(F_{1,46} = 0.084, p=0.774)$. (D) Significant increase in rearing episodes was observed in DEP treated mice compared to controls $(F_{1,46} = 4.527, *p=0.039)$. Data are presented as mean ± SEM.
Chapter 3: Preliminary studies investigating pre-natal exposure to food additives and its effect on mouse social behavior

3.1 Abstract

Autism spectrum disorders are known to have strong genetic basis. Mutations identified in individual as well as multiple genes have together contributed to its complex etiology. Studies involving twins that are used to establish heritability and contribution of genetics and environment to a certain phenotype indicate concordance rates for autism spectrum disorders to be in the range of 30% to 90%. However, a recent study indicated that concordance rates among dizygotic twins are vastly underestimated and that environmental risk factors may have a substantially bigger role in (ASD and autism) its etiology than previously known. Exposure to certain chemical compounds during pregnancy can adversely affect fetal health and is known predispose fetus to certain conditions such as intrauterine growth retardation.

We hypothesized that exposure to certain food additives during pregnancy can predispose offspring to develop impairments in social behavior. In order to test this, we added different food additives to the diet of pregnant mice. Feeding of this additive laced diet continued through gestation until weaning of offspring. The offspring were tested for autism-like behavior using a behavioral test that has face validity to one of the defining
features of autism, impaired sociability. We find that, a combination containing food colors when given during pregnancy induced behavioral changes in the offspring. These behavioral deficits, primarily a decrease in social interaction, are observed only in a subset of animals. However, consistent results were obtained when the same group of additives was tested on a different strain of mice. Findings presented in this section are preliminary and additional behavioral experiments are being done to validate them.

3.2 Background and Introduction

The recently released edition of diagnostic and statistical manual of mental disorders (DSM-5) categorizes autism spectrum disorders (ASD) into 4 separate disorders. Autistic disorder, Asperger’s disorder, childhood disintegrative disorder and pervasive developmental disorder not otherwise specified (DSM, V). Common features among all four disorders are deficits in communication and social interaction, an obsessive adherence to routine and inflexibility to changes in environment with classification reflecting severity of these symptoms.

According to the Center for Disease Control (CDC), 1 in 88 children are diagnosed with ASD (Morbidity and Mortality weekly report, Center for Disease Control). These numbers represent a 10 fold increase in prevalence rates over the past 40 years. One possible factor contributing to increase in incidences is improvement in
diagnosis and reporting, however contribution of risk factors should not be discounted. Autistic disorders are characterized by impaired social interaction, reduced social communication and restricted and repetitive behaviors. Contributing to these phenotypic features are mutations in a heterogeneous group of genes. A recent study, looking at sex-specific concordance rates in a sample of twins, concluded that environmental factors contribute to Autism etiology to a greater extent than previously reported. (Hallmayer et al., 2011).

Perinatal period is considered to be a critical time for fetal development. Interruptions during stages of neural development such as the formation of the neural tube, which occurs around gestational day 26 in humans, can lead to abnormalities of brain and spinal cord. Neurogenesis, which occurs at different rates and times in different brain regions, typically begins at gestational week 4 and continues onto to week 40 in certain brain regions (Bayer et al., 1993). Various studies have shown that exposure to environmental agents can adversely affect events such as neurogenesis, neuronal proliferation and migration. For instance, exposure to ethanol can adversely affect proliferation of neural cells in the ventricular and subventricular zone (Miller, 1996). Similarly, organophosphate pesticide exposure induces mitotic abnormalities and apoptosis in cultured rat embryo (Roy et al., 1998). These studies highlight the vulnerability of different populations of cells to environmental insults during critical periods of development. World over, there has been tremendous pressure over livestock and grain supply to feed the ever increasing population. Advances in food science
technology have allowed us to ease this burden to a certain extent by improving shelf life of food products. The Food and Drug Administration (FDA) in the United States mandates to regulate use of food additives and preservatives of which a large majority of them fall into two groups; Group I includes substances approved to be safe to use, prior to 1958 FDA amendment. Compounds in Group II are those that are approved as generally recognized as safe (GRAS) based on experience of its use in food prior to 1958 or scientific procedures (toxicology studies) used to establish their safety. This meant any additive that was in use before 1958 may be considered safe based on user experience but not on scientifically ratified safety data. A study evaluating availability of safety studies of all food additives (those that come in direct or indirect contact with food) found that many of the additives approved for use by FDA lacked reproductive and developmental toxicity studies (Neltner et al., 2013).

Our study hopes to plug some of the gaps by testing the effect of food additives on fetal behavior following exposure during gestation. We were specifically interested in autism spectrum disorders given how some of these compounds have been shown to be associated with other closely related conditions (Bateman et al., 2004, McCann et al., 2007). We hypothesized that exposure to certain commonly used food additives in addition to genetic factors could induce autism-like behavior in a subset of population. We fed pregnant mice with higher than normal levels of food additives in their diet from gestation through weaning. The rationale for using a high dose is two-fold. One, mice are less sensitive to most compounds than humans. Two, we rationalize that in the absence of
genetic risk factors, food additives by themselves and when used at the normal concentrations may not induce an autism-like phenotype. If feeding food additives at high concentrations causes behavioral changes, there is a possibility that a combined effect of genetic risk factors and normal levels of food additives would together induce a behavioral phenotype.

3.3 Materials and Methods

**Food additives** We prepared a list of 20 most commonly used food additives. Food additives were categorized into 4 classes, preservatives, colors, artificial sweeteners and vitamins. Based on the amount consumed per day in humans (estimated and accepted daily intake, Codex Alimentarius, International Food Standards, World Health Organization), relative amounts to be used in mice were calculated. For example, estimated daily intake (humans) for Ponceau 4R is 50mg/day. The equivalent dose for a mouse will be 166 mg/day calculated based on body surface normalization methods (Center for Drug Evaluation and Research, 2002, Reagan-Shaw et al., 2008). We use a dose which is about 20 fold higher than the equivalent of human consumption in order for us to detect effects of these additives in the absence of genetic predispositions. The formula for human equivalent dose (HED) is as follows, \[ \text{HED (mg/kg)} = \frac{\text{animal dose (mg/kg)} \times \text{Animal K}_m}{\text{Human K}_m} \]. Animal and human \( K_m \) values are factors calculated using the weight and body surface area. For humans the \( K_m \) value is 37 which was derived by dividing the average human body weight (60kg) by the average body surface...
area (1.6m²) and for mice, the K_m value is determined to be approximately 3 (mouse body weight (0.02g) divided by body surface area (0.007) (Center for Drug Evaluation and Research, 2002, Reagan-Shaw et al., 2008). These compounds were randomly placed in 5 different groups, with each group containing at least one preservative, sweetener, a food color and a vitamin.

**Animals** Male and female C57BL/6J and B6C3F1 mice were purchased from Jackson Labs. Animals were housed in groups of 4 with *ad libitum* access to food and water. One male and two females were placed together for breeding. As soon as the vaginal plug was spotted male and female animals were separated and females placed on a diet of additive mix. The additives were dissolved in water, soaked into standard rat chow and dried in slight vacuum overnight. Amount of additive mixed food consumed was noted every day. Feeding continued until pups were weaned at day 28. Social interaction tasks performed on animals aged 6 weeks.

**Social Interaction Tests** Social approach was measured in a 3-chambered apparatus using a previously validated procedure (Crawley, 2007). The test is divided into three 10 min stages. In the first 10 min stage, mice were allowed to freely explore the apparatus containing novel wire cages in the side chambers. This is followed by another 10 min test; wherein an unfamiliar mouse (stranger 1) is enclosed under the wire cage in one of the side chambers. The third phase involves the introduction of another unfamiliar mouse (stranger 2) which is placed under the wire cage that was previously empty. Time spent in the chambers during all three stages was measured. Cylindrical wire cages (Galaxy Cup,
Spectrum Diversified Designs, Streetsboro, OH) were used to hold the stranger mice. The location of the stranger mice and wire cage was counterbalanced between test animals. Age matched C57BL/6j mice were used as unfamiliar stranger mice and were habituated to the wire cages for 15 minutes/day for 3 days prior to the test.

3.4 Results

<table>
<thead>
<tr>
<th>Group 1 Additives</th>
<th>Group 2 Additives</th>
<th>Group 3 Additives</th>
<th>Group 4 Additives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoic acid</td>
<td>L-glutamic acid</td>
<td>Amaranth</td>
<td>Sodium Sulphite</td>
</tr>
<tr>
<td>Butylated hydroxyanisole</td>
<td>Sodium nitrite</td>
<td>Quinoline yellow</td>
<td>Sodium Nitrate</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Thiamine</td>
<td>Sunset yellow</td>
<td>Folic acid</td>
</tr>
<tr>
<td>Amaranth</td>
<td>Quinoline yellow</td>
<td>Ponceau 4R</td>
<td>Allura Red</td>
</tr>
<tr>
<td>Saccharin</td>
<td>Aspartame</td>
<td>Tartrazine</td>
<td>Sucralose</td>
</tr>
</tbody>
</table>

Table 3.1 Four groups of food additives

Food additive treatment regimen We treated pregnant mice with 4 different groups of food additives. Results from social interaction task performed on adolescent mice (6 week old) are presented below. Data are presented as social approach scores (difference in time spent in chamber containing stranger mice minus the time spent in the chamber containing an empty cage). No statistical difference in social approach score was observed between control and group 1 treated additives (Fig. 3.1A, student’s t-test t=0.6079, p=0.5472 Control (n=28), Group 1 treated (n=9)), suggesting that this combination of additives did not affect social behaviors in the offspring. Similarly, social
interaction data from male offspring of mice treated with group 2 additives is presented in Figure 3.1B. No statistical difference between control and Group 2 additive treated mice in social approach score was observed suggesting that pregnant mice when exposed to this combination of food additives did not produce offspring with social deficits (student’s t-test, t=0.5330, p=0.5974). Figure 3.2 (A and B) represent data from social approach scores of group 3 or control treated animals in two strains of mice, C57bl/6J (Fig. 3.2A) and B6C3F1(Fig. 3.2B). The experiment with group 3 additives was performed across different rounds. When data was pooled together, data comparing social approach scores between control and group 3 additives treated C57bl/6J mice did not reach statistical significance (Fig. 3.2A, student’s t-test, t= 1.154, p=0.2526, Control n=28, group 3 treated, n=44), partly because every round produced only a fraction of animals that exhibited reduced social approach score. Analyzing data from individual animals we find that 2 out of 28 controls (7%) and 8 out of 44 (18%) group 3 treated animals (data not shown) showed reduced preference for chamber containing stranger 1 (social approach score that was less than 0). This suggests that only a fraction of the population was vulnerable to the effects of the additives. This was also interesting in light of results from previous rounds (group 1 and group 2 additives) where none of the animals had social approach scores less than 0. When B6C3F1 mice were treated with group 3 additives, a significant difference was observed between controls and group 3 treated animals. (Fig. 3B, student’s t-test, t= 2.378, *p=0.02, Control n=24, group 3 treated, n=23). B6C3F1 strain mice were used in this study as they are extensively used
in cancer bioassays and in various toxicology studies. Figure 3.3C represent data from male offspring of C57Bl/6J mice treated with control or group 4 additives. A significant difference in social approach was observed between control and group 4 additive treated animals (student’s t-test, t= 2.279, *p=0.02, Control n=24, group 4 treated, n=11).

3.5 Discussion

In this preliminary study, we set out to screen changes in social behavior in offspring of mice exposed to different groups of food additives. The goal was to assess the effect of exposure to food additives during pregnancy on offspring social behavior. We find that, out of the four groups that were tested, two groups of additives produced changes in social approach scores, used here as a measure of social behavior. These results although preliminary, underline the necessity to screen FDA approved compounds to determine how they impact fetal development. These results also suggest that environmental agents such as food additives may induce deficits in social behaviors when pregnant mice are exposed to them in utero.

It has been shown previously that events during gestation can affect neurogenesis, neural proliferation, neuronal migration and synaptogenesis (Rodier et al., 1984, Slotkin et al., 1993, Ponce et al., 1994, Laev et al., 1995, Levitt, 1998). We hypothesized the certain periods of gestation are particularly vulnerable to environmental insults and events such as exposure to food additives can adversely affect brain development leading to deficits in social behaviors. The 20 compounds screened in this study were drawn
based on their occurrence in food products and consumption pattern in daily life (FAO, 1999). Each group in this study contains a combination of different classes of food additives. Group 1 and group 2 food additives did not produce any deficits in sociability in the offspring when tested using a mouse sociability task. Group 3 additives when tested on C57BL/6J animals produced a reduction in sociability albeit in fraction of animals across different rounds. In order to confirm this observation, we used the B6C3F1 strain of mice. B6C3F1 mice are routinely used in toxicology studies (Innes et al., 1969). Our laboratory had used this strain to study the behavioral effects of prenatal exposure to diesel exhaust particles (Thirtamara Rajamani et al., 2013). Exposure to group 3 additives in this strain of mice produced deficits in social approach relative to controls.

Results from both strains of mice suggest that this group of additives might induce behavioral deficits that resemble those observed in ASD. However, it is unknown if specific (group 3 additives) combinations or a single compound contributed to the observed effect. Group 3 additives contained a mix of food colors. Artificial food colors approved for use by the FDA are used in cereals, beverages and candy among other food items. In fact, there was a 5-fold increase in consumption of dyes since 1955 in the United States (CSPI, 2010). A recent meta-analyses established a causal relationship between use of food colors and hyperactivity in children with prior diagnosis of hyperactivity syndromes (Schab and Trinh, 2004). Similarly, adverse effects (hyperactivity behavior) of artificial colorings and a preservative were found in children
aged 3 with no prior history of hyperactivity (Bateman et al., 2004, McCann et al., 2007). As recently as 2008, the FDA was petitioned by Center for Science in the Public Interest (CSPI), an advocacy group, to ban certain food colors due to their carcinogenic nature. These recommendations were made following literature reviews of toxicological data of certain group of food colors. They found that most toxicological tests were dated, mostly inconclusive and ambiguous (CSPI, 2010). An FDA advisory panel was constituted and although the panel concluded that warnings labels were unwarranted given lack of scientific evidence linking food additives to hyperactivity behavior, they did acknowledge that there was insufficient scientific evidence to rule out a causal effect. A recommendation was made to carry out additional studies. Considerable controversy exists over adverse effects of food additives and preservatives. Although only correlational, a case for establishing a causal relationship is weakened by conflicting data. The data presented in this study is preliminary and interpretations be made in light of those findings. Preliminary data from our study indicates that pregnant mice exposed to food colors might induce a social deficit phenotype in a subset of offspring. It is important to note that not all animals (and across different rounds) exhibited the phenotype in both strains of mice. Although we have used the social interactions task to screen for behavior, additional tests (T-maze, social transmission of food preference) which bear face validity to other features of ASD such as repetitive, restrictive behaviors and social communication are needed to support the findings. In conclusion, we tested the effect of prenatal exposure to different food additives on mouse social behavior.
Preliminary results suggest that mice when exposed to certain food additives during pregnancy produce offspring with deficits in social behavior. These effects were observed consistently across different strains of mice. Additional studies are required to identify individual compounds that contribute to this effect and also determine the mechanism by which they induce this response. Further testing is also required to confirm if other features of ASD are affected following perinatal exposure of food additives.
Fig. 3.1 Social approach scores of animals treated with group 1 additives. (Fig 3.1A) No difference in social approach scores was observed between male offspring of control (n=28) and group 1 (n=9) additive treated animals. Fig 3.1B shows data from group 2 treated additives. Social approach scores were not different between male offspring of control and group 2 treated animals.
Figure 3.2 Social approach scores from male offspring of C57BL/J strain of mice treated with normal chow or group 3 additives is shown in Fig 3.2A. Student’s test revealed no difference in social approach scores between control and treated animals (t= 1.154, \( p=0.2526 \), Control n=28, group 3 treated, n=44). Fig 3.2B represents data from male offspring of B6C3F1 strain of mice treated with normal chow or group 3 additives. A reduction in social approach score was observed in group 3 treated animals relative to controls (student’s t-test, t= 2.378, \( *p=0.02 \), Control n=24, group 3 treated, n=23). Fig. 3.2C represents social approach score from male offspring of control (normal chow) or group 4 additive treated animals belonging to the C57BL/6J strain. A significant difference in social approach score was observed (student’s t-test, t= 2.279, \( *p=0.02 \), Control n=24, group 4 treated, n=11)
Chapter 4: Inactivation of the catalytic phosphatase domain of PTPRT/RPTPρ increases social interaction in mice

4.1 Abstract

Receptor protein tyrosine phosphatase rho (RPTPρ, gene symbol PTPRT) is a transmembrane protein expressed at high levels in the developing hippocampus, olfactory bulb, cortex and cerebellum. It has extracellular domain that interacts with other cell adhesion molecules and two intracellular phosphatase domains, one of which is catalytically active. In a recent genome wide association study, PTPRT was identified as a potential candidate gene for Autism Spectrum Disorder (ASD) susceptibility. Mutation of a critical aspartate to alanine (D1046A) in the PTPRT catalytic domain inactivates phosphatase function but retains substrate binding. We have generated a knockin mouse line carrying the PTPRT D1046A mutation. The D1046A mutation in homozygous knockin mice did not significantly change locomotor activities or anxiety-related behaviors. In contrast, male homozygous mice had significantly higher social approach scores than wild-type animals. Our results suggest that PTPRT phosphatase function is important in modulating neural pathways involved in mouse social behaviors relevant to the behavioral features of human ASD patients.
4.2 Background and Introduction

Receptor Protein Tyrosine Phosphatase rho (RPTPρ; gene symbol PTPRT), is a transmembrane molecule expressed at high levels in the mouse brain during development (McAndrew et al., 1998a, McAndrew et al., 1998b). Over the past decade, PTPRT gene structure has been characterized (Besco et al., 2001, Besco et al., 2004), its expression pattern in developing mouse brain determined (McAndrew et al., 1998a, McAndrew et al., 1998b) and several substrates identified (Besco et al., 2006, Zhang et al., 2007, Zhao et al., 2010, Park et al., 2012). The PTPRT gene was mapped to human chromosome 20q12-13.1 and to a syntenic region on mouse chromosome 2 (McAndrew et al., 1998b).

It is classified as a receptor protein tyrosine phosphatase (RPTP) of the type 2B subfamily, together with RPTPK, RPTPM and PCP-2 (Gebbink et al., 1991, Wang et al., 1996, Yang et al., 1997, Tonks, 2006). The RPTPρ protein contains an extracellular segment, featuring a MAM domain, an immunoglobulin-like domain, four fibronectin type II repeats, a transmembrane domain, and an intracellular segment. The latter contains two highly conserved phosphatase domains, only the first of which is catalytically active (Fischer et al., 1992). Motifs in the extracellular ectodomain interact with identical conserved segments on other molecules, whereas the intracellular segment facilitates signal transduction through protein dephosphorylation via the catalytic first phosphatase domain (Brady-Kalnay et al., 1993, Johnson and Van Vactor, 2003, Besco et al., 2006).

A recent analysis of a large, extended autism pedigree identified PTPRT/RPTPρ
as a potential candidate for autism susceptibility (Allen-Brady et al., 2009). Autism Spectrum Disorder (ASD) is a complex, heterogeneous disorder with variations in multiple genes likely contributing to its etiology (Kumar and Christian, 2009). Numerous genes identified as risk factors for ASD are involved in maintaining synaptic function, indicating that altered synaptic homeostasis is an important underlying factor in ASD pathogenesis (Betancur et al., 2009, Huguet et al., 2013). In vitro studies in hippocampal neurons have shown that altering PTPRT expression affects dendritic arborization (Park et al., 2012) and the number of spines and synapses (Lim et al., 2009).

It is possible that PTPRT may regulate neuronal pathways involved in animal behaviors relevant to ASD by modulating proteins important in synapse formation and synaptic transmission. Substitution of the critical aspartate 1074 to alanine in human PTPRT in the first phosphatase domain inactivates its phosphatase activity but retains substrate binding (Blanchetot et al., 2005, Zhang et al., 2007, Zhao et al., 2010). We have generated a knockin mouse carrying a mutated PTPRT (D1046A) that is equivalent to the human D1074A mutation. We have conducted behavioral analyses of these mice to determine if inactivation of PTPRT phosphatase function has an impact on mouse behaviors relevant to behavioral symptoms seen ASD patients.

4.3 Materials and Methods

*Generation of the RPTPp knockin mouse* A *ptpr*<sup>D1046A</sup> knockin mouse line was generated using procedures similar to those employed previously in our laboratories (Wu
and Gu, 2003, Chen et al., 2005, Chen et al., 2006a, Chen et al., 2006b, Gu et al., 2006).

Briefly, a targeting construct was assembled by joining the long and short homology arms, a positive selection marker (neomycin resistant gene) flanked by two loxP sites, and a negative selection marker (thymidine kinase gene) (Figure 3.1). The homology arms were PCR amplified using a BAC clone of C57BL6J mouse genomic DNA as the template (RP23-466K24, Children’s Hospital, Oakland). The short arm was 1.3 kb and the long arm was a 5.5 kb fragment of the PTPRT gene containing exons 24 and 25.

Three point mutations were introduced in Exon 25, one introduced the D1046A mutation and the other two were synonymous introducing a Spe1 restriction site nearby for construct assembly and diagnosis. The linearized targeting construct was electroporated into mouse ES cells (129 SvJ). G418-resistant ES cell clones were analyzed by PCR. A clone displaying the desired homologous recombination was microinjected into blastocysts of C57BL6J mice and implanted into pseudo pregnant C57BL6J foster female mice at the OSU Genetically Engineered Mouse Modeling Core. Chimeric mice were bred with C57BL6J mice and germ line-transmitting mice were identified by PCR.

Wild-type (+/+), heterozygous (+/m) and homozygous (m/m) knockin mice used in the experiments were genotyped by PCR using primers that amplify the regions around the Neo cassette insertion site. Tissue from the cerebellum (from each genotype) was dissected and disrupted using a polytron tissue homogenizer and mRNA was isolated using an mRNA isolation kit (Qiagen Corp). The cDNAs were synthesized and the mutated region in the PTPRT cDNA was PCR amplified for all three genotypes. The
PCR products were sequenced by the OSU Comprehensive Cancer Center Nucleic Acid Shared Resource.

**Behavioral Assays**

Wild-type (+/+) heterozygous (+/m) and homozygous (m/m) PTPRT knockin mice were used in the following behavioral experiments. Data from heterozygotes was, in general, similar to that of wild-type and is not shown.

*Open field locomotion* Spontaneous locomotor activity was measured in a 40 cm x 40 cm in an acrylic box placed in a sound-attenuating chamber for 20 minutes (Fonken et al., 2011, Brielmaier et al., 2012). Total distance travelled was measured using tracking software (ANYmaze, Stoelting Co., Wood Dale, IL)

*Elevated plus maze* The elevated plus maze apparatus consisted of an elevated cross, placed 24cm above the floor, with two open arms (35x6cm²) and two closed arms (35x6x22cm³), at right angles to each other. Mice were placed at the intersection of the two arms and allowed to explore the maze for 5 minutes (Holmes et al., 2003). Time spent in the open versus closed arms and the number of entries into each arm was recorded and analyzed using the ANYmaze video tracking system. Arm entry was defined as the placement of all fours limbs into the arm.

*Social Interaction Task* Social approach was measured in a 3-chambered apparatus using a procedure as previously described (Thirtamara Rajamani et al., 2013), and similar to the procedure developed by Crawley et al (Crawley, 2004). Briefly, the test was divided into three 10 min stages. During the first 10 min, mice were allowed to explore the apparatus.
This was followed by another 10 min stage, wherein an unfamiliar mouse (Stranger 1) was placed under a wire cage in one of the side chambers, along with an empty wire cage in the opposing chamber. The third stage involved the introduction of a second unfamiliar mouse (Stranger 2), which was placed under wire cage in the opposite side chamber. Movement was recorded with the video tracking system; time spent in each chamber during all three stages was measured. Cylindrical wire cages (Galaxy Cup, Spectrum Diversified Designs, Streetsboro, OH) were used to hold the stranger mice. The placement of the stranger mice in the side chambers was counterbalanced among test animals to minimize any initial preference of the side chambers. Age and sex matched C57BL/6J mice were used as unfamiliar stranger mice and were habituated to the wire cages for 15 minutes/day for 3 days prior to the test.

4.4 Results

Confirmation of RPTPρ knockin mouse

The D1046A mutation was introduced into the mouse RPTPρ gene by standard homologous recombination in mouse ES cells. The general targeting strategy is described in Figure 4.1. To confirm the presence of the desired mutations, mRNAs were isolated from the cerebellum of adult wild-type, heterozygous and homozygous knockin mice and cDNA was synthesized. A region of the RPTPρ cDNA containing exon 25 was amplified by PCR and sequenced. The sequencing reactions for the wild-type and homozygous mice produced a clean sequencing chromatogram with single peaks (data not shown); the
sequences were as expected for the wild-type and mutated alleles. The sequencing chromatogram for heterozygous mice showed double peaks at the mutation sites, indicating the presence of both wild-type and mutated alleles. Importantly, the heights of the two peaks at the 3 mutation sites were approximately the same; this was confirmed with an additional heterozygous mouse. These results indicate that the mutations and presence of the Neo cassette in the intron between exons 25 and 26 in the mutant allele did not change the mutant RPTP\(\rho\) mRNA expression level; therefore, we did not remove the Neo cassette.

Open field locomotor activity

Wild-type (+/+) and Homozygous (m/m) mutant PTPRT KI mice were placed in an open field box and their locomotor activity was recorded. Total distance traveled by male and female mice, and the distance traveled in 5 minute bins throughout the 20 minute were measured. A one-way ANOVA revealed no differences in total distance traveled in male (Fig. 4.2A, \(F_{1,18} = 2.354, p=0.143; +/+ n=8, m/m n=11\)), and female (Fig. 2C, \(F_{1,29} = 0.036, p=0.85; +/+ n=13, m/m n=17\) PTPRT knockin mice. A repeated measures ANOVA revealed a significant decrease in distance traveled over time for both genotypes, suggesting habituation to the open field apparatus (Males, Fig. 4.2B, \(F_{3,18} = 6.534, p<0.05\); Females, Fig 4.2D, \(F_{3,29} = 35.114, p<0.05\)). Male m/m knockin mice were slightly, but not significantly, less active than wild-type controls at each 5 minute data point throughout the 20 minute period.
Elevated plus maze

Animals were placed in the center of an elevated plus maze with two open arms and two closed arms. Mice were monitored for 5 minutes and the number of entries and time spent on the open arm of the maze was measured. No statistically significant difference between the two genotypes was observed in the number of entries into the open arms in males (Fig. 4.3A, Kruskal-Wallis Test, p=0.706, unequal variances observed using Levene’s Statistic, p=0.027) or females (Fig. 4.3C, One-way ANOVA, $F_{1,29} = 0.833$, $p=0.369$). This result suggests that exploratory behavior does not differ between the two groups. Time spent in the open arms of the maze also did not differ significantly between $+/+$ and m/m mice in both males (Fig. 4.3B, $F_{1,18} = 0.053$, $p=0.822$) and females (Fig. 4.3D, $F_{1,29} = 0.006$, $p=0.938$), suggesting that homozygous knockin mice did not display an anxiogenic phenotype.

Social Interaction Tests

Social Interaction was tested using a 3-chambered apparatus. In the first 10 minute period of this 3 part test, mice were allowed to explore the apparatus with empty cages in both side chambers; each group showed no preference for either of the two side chambers (data not shown).

Social Approach: In the second period, a stranger mouse (Stranger 1) was placed under a cage in one of the side chambers; the alternate side chamber contained an empty wire cage. Test mice were allowed to explore for 10 minutes. Both male (Fig. 4.4A) and
female (Fig. 4.4B) PTPRT +/+ and m/m mice spent more time in the chamber containing Stranger 1 than with the empty wire cage (male +/+, one-way ANOVA, $F_{1,44} = 12.818$, $p<0.05$; male m/m, $F_{1,44} = 37.867$, $p<0.05$; female +/+, $F_{1,44} = 6.658$, $p<0.05$; female m/m, $F_{1,44} = 6.282$, $p<0.05$), demonstrating the tendency for social interaction. Social approach scores, defined as the difference in time spent between the chamber containing Stranger 1 and the chamber containing the empty wire cage, were also analyzed. Male PTPRT m/m (Fig. 4.4C, n=8) mice had a significantly higher social approach scores than +/+ (n=16) mice (student’s t-test, $t=2.157$, $p<0.05$), suggesting that male PTPRT m/m mice are significantly more socially inclined than their wild-type counterparts. However, no difference in social approach scores was observed between female +/+ (n=13) and m/m (n=11) PTPRT mice (Fig. 4.4D) (student’s t-test, $t=0.07025$, $p=0.9446$).

Social Novelty: In the third stage of the social interaction test, Social Novelty, a novel stranger mouse (Stranger 2) was placed under the previously empty wire cage in the side chamber, while the Stranger 1 mouse remained in the alternate compartment. Test mice were allowed to explore for 10 min and time spent in each of the three chambers was recorded. In general, male (Fig. 4.5A) and female (Fig. 4.5B) mice of both genotypes preferred to spend more time in the chamber containing Stranger 2 than with Stranger 1 (male +/+, one-way ANOVA, $F_{1,44} = 18.287$, $p<0.05$; male m/m, one-way $F_{1,44} = 12.220$, $p<0.05$; female +/+, $F_{1,44} = 12.43$, $p<0.05$; female m/m, $F_{1,44} = 0.693$, $p = 0.41$). The social novelty scores (defined as difference between times spent in the chambers containing Stranger 2 and Stranger 1 respectively) were also measured. The male wild-type and
m/m mice had similar social novelty scores (Fig. 4.5C; $F_{1,23} = 0.085$, $P=0.773$), while female m/m mice showed a lower social novelty score than the wild-type group. However, the difference was not significant (Fig. 4.5D; $F_{1,23} = 1.653$, $P=0.212$).

4.5 Discussion

Protein tyrosine phosphatase rho (RPTPp/PTPRT) is a transmembrane protein expressed predominantly in the central nervous system (CNS) (Paul and Lombroso, 2003), where it plays a role in signal transduction (Xu and Fisher, 2012), cell adhesion (Sallee et al., 2006) and synaptogenesis (Lim et al., 2009). Improper synapse formation can contribute to neurodevelopmental disorders and many genes identified in the pathogenesis of ASD are associated with synaptogenesis (Zoghbi and Bear, 2012). We have generated a knockin mouse line carrying a mutated PTPRT with the phosphatase function inactivated.

We speculate that this could lead to increased phosphorylation states of PTPRT substrate proteins thus affecting neural pathways mediating certain animal behaviors. We have found that inactivating the phosphatase function of PTPRT in the homozygous knockin mice significantly increased the social approach score compared to the wild-type mice. The performances of homozygous knockin mice on locomotor tests and anxiety-related tests were generally unchanged compared to wild-type mice, ruling out the possibility that altered locomotor activity could significantly impact the outcome of the Social Interaction tasks.
Although PTPRT has been shown to play a critical role as a colorectal tumor suppressor (Wang et al., 2004), its functional role in the central nervous system is only now emerging. Unlike other type 2B RPTPs, PTPRT expression is prevalent in the central nervous system (McAndrew et al., 1998b), where it is expressed at high levels in the cortex, hippocampus, olfactory bulb and cerebellum during the peak of synapse formation and is downregulated thereafter to lower adult levels (McAndrew et al., 1998a, McAndrew et al., 1998b). Recent in vitro studies in hippocampal neurons have shown that upregulation of PTPRT expression results in increased dendritic spine density, and number of excitatory and inhibitory synapses (Lim et al., 2009).

When PTPRT was knocked down, dendritic arborization and number of spines was reduced (Park et al., 2009). More recently, PTPRT has been shown to dephosphorylate tyrosine 145 of Syntaxin-binding protein 1 thereby regulating its interaction with Syntaxin-1 (Lim et al., 2013), a member of the SNARE complex, which is necessary for fusion of synaptic vesicles with the presynaptic membrane (Jahn and Sudhof, 1999). RPTPρ is also known to interact with neuroligin and protein tyrosine kinase, Fyn, to regulate synapse formation (Lim et al., 2009). Mutations in both neurogulins (NL-3 and NL-4) and neurexin-1 have been reported in a subset of patients with ASD (Yan et al., 2005, Kim et al., 2008). Together, the above studies strongly suggest that PTPRT may play a regulatory role in synaptogenesis.

Autism spectrum disorders are complex polygenic disorders. Genome wide analysis studies have identified multiple genes associated with ASD, many of which are
involved in synapse formation and synaptic transmission (Jamain et al., 2003, Durand et al., 2007). The three core diagnostic symptoms of ASD (impaired sociability, deficits in social communication and repetitive and restrictive behaviors (American Psychiatric Association, 2013) have been modeled in animals (Crawley, 2007, Ricceri et al., 2007).

We tested PTPRT mutant mice for deficits in social behaviors using the 3-chambered Social Interaction test. This test has been extensively studied and employed for screening social deficits in various mouse models of autism (Moy et al., 2007, McFarlane et al., 2008, Moy et al., 2008, Silverman et al., 2011).

Animal models of autism have been reported with deficits in Social Approach (DeLorey et al., 2008, Jamain et al., 2008, Molina et al., 2008, Moy et al., 2009, Nakatani et al., 2009). We hypothesized that inactivating phosphatase function of PTPRT might shift the balance of phosphorylation/dephosphorylation states of proteins critical for synapse formation resulting in altered neural pathways and animal behaviors relevant to ASD. Indeed, male homozygous PTPRT m/m knockin mice initiated more contact with the Stranger 1 (Social Approach) mice than their wild-type controls, suggesting increased, rather than decreased social interaction.

Although the precise neuronal pathways underlying these findings are unknown, it is well understood that tyrosine phosphatases serve as signal transducing agents for proteins involved in synaptic development (Dabrowski and Umemori, 2011). Reduced PTPRT phosphatase function is likely to result in higher phosphorylation states of substrate proteins, disrupting the balance of phosphorylation/dephosphorylation in critical
pathways and potentially modifying behaviors. Our results indicate a possible role for PTPRT/RPTPρ in shaping neural pathways important for specific social behaviors. Further studies are required to better understand RPTPρ’s involvement in mouse behaviors relevant to ASD.
Figure 4.1 Targeting strategy for generating PTPRT knockin mouse. Thin lines represent wild-type mouse genomic DNA between exons 23 and 26; thick lines represent sequences included in the targeting construct. Open boxes represent exons; the large box represents exon 25, which contains a line representing the mutation in exon 25. Open arrows represent the positive selection marker (Neo cassette) and the negative selection marker (thymidine kinase gene). Triangles represent LoxP sites.
Figure 4.2 Open field locomotor activity. The total distance traveled in 20 minutes by male and female wild-type (+/+) and homozygous PTPRT mutant mice (m/m) are shown. No significant difference in the total locomotor activity was observed between +/+ and m/m mice (Fig. 4.2A, C). Distance traveled over time is shown for males (Fig. 3.2B) and females (Fig.4.2D); a significant effect of time, but no effect of genotype was observed. Data are presented as mean ± SEM.
Figure 4.3 Elevated plus maze. The number of entries into the open arms are shown for male (Fig. 4.3A) and female (Fig. 4.3C) wild-type (+/+ ) and homozygous PTPRT mutant mice (m/m). Time spent on the open arms for males and females is shown in Figs. 4.3B and 4.3D. Data are presented as mean ± SEM.
Figure 4.4 Social Approach. Time spent in the center chamber, the side chamber containing an unfamiliar mouse (Stranger 1), or the side chamber containing an empty wire cage for male (Fig. 4.4A) and female (Fig. 4.4B) wild-type (+/+) and homozygous PTPRT mutant mice (m/m). In males, both genotypes spent significantly higher time in the chamber containing stranger 1 than wire cage (*p<0.05). Social Approach scores (Fig. 4.4C) were significantly higher in male m/m mice (n=8) compared to +/+ (n=16) mice (*p<0.05). Both +/+ and m/m female mice showed preference for the chamber containing stranger 1 over wire cage (*p<0.05). No differences in social approach scores were observed between female +/+ (n=13) and m/m (n=11) mice (Fig. 3.4D). Data are presented as mean ± SEM.
Figure 4.5 Social Novelty. Time spent with Stranger 1 and 2 for male (Fig. 4.5A) and female (Fig. 3.5B) wild-type (+/) and homozygous PTPRT mutant mice (m/m). In males, both +/+ and m/m mutant mice spent significant amount of time with stranger 2 than stranger 1 (*p < 0.05). In females, only +/+ and not m/m mutant mice, showed significant preference for chamber containing stranger 2 over stranger 1 (*p < 0.05). Figs. 4.5C and 4.5D show the difference in time spent with Stranger 2 vs. Stranger 1 (social novelty score) by male (C) and female (D) +/+ and m/m mice. Data are presented as mean ± SEM.
Chapter 5: Introduction to animal models of Cocaine Addiction

The health-related cost of drugs of abuse in the United States was $180.9 billion in 2002, of which about $107.8 billion is attributed to crime-related costs (Policy, 2004). Drugs of abuse can be broadly and predominantly classified into opiates, cannabinoids, ethanol, nicotine and psychostimulants. Cocaine abuse is a serious socioeconomic problem in the United States, contributing to more than half the instances of healthcare sought compared to other drugs of abuse. According to the Food and Drug Administration (FDA), “abuse potential” of any drug is defined by its ability to induce “a positive psychoactive state” in the form of euphoria, sedation and hallucination and be used repeatedly and/or sporadically. Repeated use/abuse of such drugs can ultimately lead to “addiction”.

Addiction is defined as a state of uncontrolled drug use characterized by intense craving, compulsive and repeated use despite negative consequences (Leshner, 1997). This aspect of addiction is attributed in part to action of drugs of abuse on the so-called and brain reward pathway. Central to this “pathway” is the mesolimbic dopamine system. The pathway is part of an evolutionary primitive neuroanatomical brain substrate that is known to mediate behaviors such as sex and feeding; behaviors critical for survival of a species. The earliest study that attributes the mesolimbic pathway in mediating reward-
related behaviors came from Olds and Milner. Rats would increase their response on a lever in order to receive an electrical stimulation of the median forebrain bundle (MFB) (Olds and Milner, 1954). Cocaine produces a similar response to electrical stimulation of MFB leading to the hypothesis that cocaine simply usurps the existing system in place. However, mechanistically the process of reward (natural) and addiction are different, with the only overlapping feature being brain regions associated with each of these phenomena. Mesolimbic pathway consists of dopaminergic neurons originating in the ventral tegmental area (VTA) that project to structures such as nucleus accumbens (in the ventral striatum), amygdala, hippocampus and the lateral hypothalamus.

Cocaine’s role in activating this pathway was first demonstrated by Di Chiara, who showed that cocaine administration increased dopamine release in the nucleus accumbens (Di Chiara and Imperato, 1988). Subsequently many studies have shown nucleus accumbens to play a critical role in mediating cocaine’s rewarding effects. (Roberts et al., 1980, Maldonado et al., 1993). Nucleus accumbens itself is not anatomically uniform region and is further subdivided into shell and core. These regions of the nucleus accumbens receive divergent inputs and are known to be functionally different too.

**Dopamine Receptor Signaling**

Dopamine signaling is mediated by a group of seven transmembrane G protein-coupled receptors paired into two classes. Dopamine D1-like receptors include D1 and
D5 receptors and dopamine D2-like receptors include D2, D3 and D4 receptors (Civelli et al., 1992). Upon activation, D1-like receptors activate adenylyl cyclase which in turn catalyzes conversion of ATP to cyclic AMP (cAMP). This in turn activates protein kinase A (PKA) which subsequently regulates expression of a variety of genes. D2-like receptors on the other hand, negatively regulates adenylyl cyclase leading to inhibition of cAMP. Receptor distribution of these two classes of receptors is also distinct.

D1 receptors are widely distributed in the dorsal striatum, ventral striatum and olfactory tubercle (Missale et al., 1998). Receptor expression has also been reported in substantia nigra, ventral tegmental area, amygdala, hippocampus and prefrontal cortex (Hall et al., 1994, Jackson and Westlind-Danielsson, 1994). Relative to D1 receptors, D5 receptors are scarcely distributed in the brain with expression restricted to hippocampus, prefrontal cortex, hypothalamus and ventral striatum (Choi et al., 1995, Khan et al., 2000). D2 receptor expression has also been reported in the hypothalamus, thalamus and pituitary gland (Weiner et al., 1991, Hurd et al., 2001). D3 receptors are found in the ventral striatum, hippocampus and the islands of celleja whereas D4 receptors are expressed in the amygdala, hippocampus, hypothalamus and the frontal cortex (Lahti et al., 1995, Defagot and Antonelli, 1997, Suzuki et al., 1998).

Dopamine D1 and D5 Receptor Signaling in Cocaine Addiction

Pharmacological studies suggest that D1-like receptors are likely to mediate cocaine’s discriminative and reinforcing effects. For example, animals trained to
discriminate saline from cocaine in a two lever paradigm showed decreased lever pressing behavior in the presence of a dopamine D1-like antagonist (Barrett and Appel, 1989). Similarly, rhesus monkeys have been shown to self-administer non-selective D1-like receptor agonists (Weed and Woolverton, 1995). Although these studies appear to indicate a role of D1-like receptors in mediating cocaine’s effects, no distinction is made between D1 or D5 receptors. This is because most agonists bind to and activate both receptors.

On the other hand genetic studies have indicated a mixed role for D1-like receptors in mediating cocaine’s effects. To begin with, D1 receptor knockout mice show increased levels of mid brain dopamine levels in addition to reduced levels of dynorphin and substance P (Drago et al., 1994, El-Ghundi et al., 1998). D1R knockout mice also have elevated basal locomotor activity and show impaired motor coordination (Cromwell et al., 1998, Karasinska et al., 2005). D1R KO mice also respond differently to locomotor stimulating effects of a D1-like agonist. Some studies show an attenuation of locomotor activity in response to D1-like agonist whereas show the opposite (Smith et al., 1998, Dracheva et al., 1999, Karasinska et al., 2000). However, D1R KO show normal conditioned place preference to cocaine, although self-administration is attenuated (Miner et al., 1995, Caine et al., 2007). Cocaine induced hyperactivity as well as sensitizing effects are attenuated in D1R knockout mice (Karlsson et al., 2008).

There is very little research investigating involvement of D5Rs in mediating cocaine’s effects. Using antisense oligonucleotides, a study had shown that D5Rs in the
nucleus accumbens are necessary for the discriminative effects of cocaine, however D5R knockout mice respond normally to acute and sensitizing doses of cocaine and show place preference behaviors (Filip et al., 2000, Karlsson et al., 2008). Taken together, these results suggest that in order to study D1-like receptors in the context of cocaine addiction better tools are necessary, tools that are capable of discriminating D1Rs from D5Rs.

In this study we intend to address the following question. Do D1R and D5Rs expressed in the nucleus accumbens have separate roles in mediating the rewarding and reinforcing effects of cocaine? Using a viral mediated shRNA system will help us differentiate their roles as well identify associated brain regions that may be involved.
Chapter 6: Dopamine D1 receptors in the nucleus accumbens contribute to cocaine’s acute and chronic locomotor effects

6.1 Abstract

Cocaine binds to and inhibits three different transporter proteins namely norepinephrine, serotonin and dopamine transporter. Inhibition of dopamine transporter leads to an increase in levels of dopamine within the extracellular space. Downstream signaling effects of dopamine are mediated by two families of G-protein coupled receptors; D1-like which consists of D1 and D5 receptors (D1R and D5R) and D2-like which include D2, D3 and D4 receptors. D1R are expressed highly in the dorsal and ventral striatum, prefrontal cortex, olfactory tubercle, amygdala and thalamus; whereas D5R are expressed in low numbers in the ventral striatum and relatively high concentrations in hippocampus and subthalamic nucleus.

Compensatory adaptations in receptor knockout models and the lack of ligands that distinguish D1R from D5R have limited our understanding of these receptors in mediating cocaine’s effects. In order to overcome these limitations, we used AAV viruses expressing short hairpin RNA’s against D1R or D5R to knockdown their expression in the nucleus accumbens (NAc), a brain region within the basal ganglia that has been central to cocaine’s actions. We report no changes in basal locomotor activity in either
D1R or D5R knockdown animals. Cocaine induced locomotor stimulation was reduced in D1R knockdown animals, but was unaffected in D5R knockdown animals compared to animals injected with control virus. D5R knockdown animals showed locomotor sensitization induced by repeated cocaine whereas D1R knockdown animals failed to sensitize to repeated injections of cocaine. We also observed a trend toward reduced conditioned place preference to cocaine in D1R knockdown animals; while D5R knockdown animals exhibited normal cocaine induced conditioned place preference. Taken together, these findings suggest that D1R and D5R expressed within the nucleus accumbens have distinct roles in mediating cocaine’s effects.

6.2 Background and Introduction

Mechanisms underlying addictive properties of most drugs of abuse are not fully understood and are different for every drug. Considerable progress has been made in treating addiction associated with drugs of abuse such as heroin and nicotine; however treating options for cocaine addiction are scarce.

Cocaine binds to and inhibits dopamine, norepinephrine and serotonin transporters causing an increase in levels of dopamine, norepinephrine and serotonin in the extracellular space of brain regions expressing these transporters (Ritz et al., 1987, Reith et al., 1997). Downstream signaling following cocaine’s inhibition of dopamine transporter is mediated by two families of seven-transmembrane G protein-coupled receptors; D1-like and D2-like. D1-like includes D1 (D1R) and D5 receptors (D5R) and

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D2-like includes D2, D3 and D4 receptors. They are classified based on their ability to regulate the activity of adenylyl cyclase. D1R and D5R receptors are expressed in varying quantities and diverse brain regions such as the olfactory bulb, prefrontal cortex, dorsal striatum, ventral striatum, amygdala and to a lesser extent in the hippocampus (Weiner et al., 1991, Beaulieu and Gainetdinov, 2011). D1R are expressed in high numbers in the nucleus accumbens, whereas expression of D5R is considered to be low. (Seeman and Van Tol, 1994, Missale et al., 1998, Khan et al., 2000). The nucleus accumbens which along with olfactory tubercle constitutes the ventral striatum, receives dopaminergic neurons projecting out of the ventral tegmental area (VTA), one of the few regions in the midbrain that contain dopaminergic cell bodies, besides substantia nigra and arcuate nucleus of hypothalamus (Ungerstedt, 1971).

Dopaminergic cell bodies originating in the substantia nigra (SNC) project to dorsal striatum. This constitutes the nigrostriatal pathway. Traditionally mesolimbic pathway has been associated with mediating addiction and reward-related functions and the nigrostriatal pathway with motor behavior. For instance, in Parkinson’s disease there is selective degeneration of neurons in the nigrostriatal pathway (Davie, 2008). On the other hand, earliest evidence for involvement of mesolimbic pathway in reward-related behaviors come from studies that show disruption of cocaine self-administration following infusion of 6-hydroxydopamine (6-OHDA) into the nucleus accumbens (Zito et al., 1985) and injections of dopamine D1 receptor antagonists of nucleus accumbens.
blocking cocaine self-administration in rats (Robledo et al., 1992). However, a likely role for SNC dopaminergic neurons in reward behaviors has been proposed (Wise, 2009).

A role for D1-like receptors in mediating cocaine responses was first described when antagonism of D1-like receptors blocked the discriminative stimulus effects of cocaine. (Woolverton and Virus, 1989). Most studies that report involvement of dopamine D1 receptors in mediating cocaine’s effects do so without essentially separating D1R from D5R. This is because of lack of ligands that can distinguish the two. Although gene knockout studies have been helpful in shedding light on their function, certain behavioral anomalies possibly from developmental compensation have obscured interpretation of results (Dracheva et al., 1999, Waddington et al., 2005). Gene knockout models are also limited by alteration in gene expression “globally”, effectively limiting our knowledge of how specific brain regions mediate function.

In order to overcome these limitations, we designed viral vectors expressing short hairpin RNA (shRNA) against D1Rs and D5Rs respectively. This enables us to alter expression of either D1R or D5R in a brain region specific manner. The focus of this study was to outline the roles of D1R and D5R in the nucleus accumbens in mediating cocaine’s effect on reward and locomotion. Delineating the function of specific receptor sub-types would enhance our understanding of the molecular mechanisms mediating cocaine’s effects.

6.3 Materials and Methods

Screening for effective shRNA
Selection of effective shRNA was performed using a technique described previously (Naughton et al., 2011). A target plasmid was constructed by inserting cDNA of D1R or D5R into the 3’ untranslated region of a green fluorescent protein (GFP) expression cassette (GFP-D1R or GFP-D5R). The construct was designed in a manner such that the target cDNA (D1 or D5R) sequence is placed between the stop codon of GFP transcript and a poly(A) tail. This way the construct gets transcribed as a single transcript, but translation is restricted to GFP alone due to placement of stop codon. Various shRNA’s targeting different regions of D1R or D5R mRNAs were designed using siRNA wizard (Invivogen). Each of these shRNA’s were then cloned into a ptripZ shRNAAmir vector (Open Biosystems, Huntsville, AL), along with a red fluorescent protein (RFP).

The presence of RFP allows us to determine expression levels of the shRNA construct itself. Both constructs, cDNA (of D1 or D5R) fused to GFP and shRNA constructs fused to RFP were co-transfected into chinese hamster ovary (CHO) cells. Once shRNA construct recognizes the complementary sequence it begins to degrade it. During this process GFP is also degraded as cDNA of D1R or D5R was inserted into the 3’ untranslated region of GFP. Fluorescent images were taken at 24, 48 and 72 hours and fluorescent intensity quantified using ImageJ software (National Institute of Health, Bethesda, MD). A reduction in fluorescence (GFP) intensity indicated successful knockdown of target transcript. shRNA constructs with highest knockdown as tested in vitro were packaged into adeno associated viral vectors serotype 1(AAV-1) at the OSU viral vector core. Additionally, a shRNA against GFP (AAVshGFP) was cloned and
packaged into viral vector. This served as negative control for the D1 (AAVD1shR) and D5 shRNA (AAVD5shR) viral vectors.

**Animals** 6 weeks old C57 BL/6J mice were obtained from Jackson labs and housed in polycarbonate cages (28 x 17 x 12 cm) with constant temperature (21 ± 2º C) and humidity (60 ± 5%) with *ad libitum* access to food and water under a 12 hour light-dark cycle. Prior to surgery mice were allowed to habituate to the vivarium for two weeks. All procedures used were approved by The Ohio State University Institutional Animal Care and Use Committee.

**Viral Vector Injection** A mixture of ketamine (JHP Pharmaceuticals, Parsippany, MI) and Xylazine (Sigma-Aldrich, St. Louis, MO) was used to anesthetize mice. A small incision was made in the skull area and mouse fixed to the stereotaxic frame. The skull was leveled and bregma was marked. A small hole was drilled into the skull at the appropriate coordinate using a power drill (Dremel, Mt Prospect, IL). A syringe pump (KD Scientific, Holliston, MA) was used to pump the virus which was primed and loaded into PE50 tubing connected to a 50 uL Hamilton syringe on one end and a injector cannula (OD: 33 GA, Plastics One) on the other. 1-2ul of virus was injected into the mouse brain at a rate of 0.1ul/min. The viral vectors were injected into the lateral nucleus accumbens using the coordinates, A-P +1.5, M-L ±1.2, D-V -4.6 at a 12º angle.

**In vivo knockdown assay** Viral vectors (AAVshGFP, AAVD1shR or AAVD5shR) were injected bilaterally into the lateral nucleus accumbens. Following three weeks of recovery, mice were sacrificed by cervical dislocation and 1mm hole punched tissue was
taken from (approximate) injection site. RNA quantitation was performed using procedures described previously (Naughton et al., 2012). Briefly, tissue from both hemispheres was combined and RNA extracted using RNAeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized by performing a reverse transcriptase reaction using the iscript cDNA synthesis kit (Thermo Scientific, Waltham, MA). Quantitative PCR using Taqman probes (Sigma Aldrich) was performed. The primers were tagged to fluorophore probes such as Fam or Hex. A multiplex qPCR was performed on a Mastercycle realplex (Eppendorf, Westbury, NY).

**Drugs** Cocaine Hydrochloride was obtained from the NIDA drug supply program. Drug was administered at 10mgkg (10ul/g) intraperitoneally (i.p) by dissolving in 0.9% saline. D1-like agonist SKF81297 was purchased from Tocris Bioscience, Bristol, UK.

**Conditioned Place Preference** Conditioned place preference (CPP) was performed using a method that was previously described (Shimosato and Ohkuma, 2000). CPP was performed in a 3 chambered apparatus. Prior to starting the experiment, mice were habituated to experimenter handling for 3 days. The test is divided into 3 parts; pretest, conditioning phase and posttest. Day one of the test, mice were allowed to explore an apparatus in which all three chambers were made distinct using tactile and visual cues. Time spent in each of the 3 chambers was recorded using a tracking device (ANYmaze software, Stoelting, Wood Dale, IL). This constituted the “pretest”. The next part of the test was the conditioning phase. During this phase, mice were injected with saline or cocaine on alternating days and placed in the apparatus consisting different cues sets on
saline and cocaine days. A total of 4 pairings (saline-cocaine) were performed. The last part of the test is the posttest which was performed immediately after the last pairing. In this part, mice were placed in an apparatus maintained in similar environment (cues) as pretest. The animal was allowed to explore the box for 30 minutes and time spent and locomotor activity measured.

**Immunohistochemistry** Animals were perfused with 4% paraformaldehyde and placed in 30% sucrose overnight. 30um tissue sections were cut using a freezing microtome. Endogenous peroxidase activity was inhibited by incubating tissue in 30% hydrogen peroxide and 10% methanol mixture for 20 minutes. Tissue sections were then blocked with 5% goat serum (Sigma Aldrich, St. Louis, MO) for an hour. A turboRFP (Evrogen) primary antibody (1:500) dissolved in PBS-T was then added to the tissue and incubated overnight at 4C. Sections were washed with PBS and a goat anti rabbit (1:200) (Sigma Aldrich, St. Louis, MO) for 1 hour. A rabbit PAP (1:200) (Jackson ImmunoResearch, West Grove, PA) was added to the tissue and the staining developed using the DAB technique.

**Statistical Analysis** Statistical analysis was performed using SPSS (SPSS 21.0). Two-way ANOVA was used to analyze results from locomotor activity. Repeated measures ANOVA were used for locomotor sensitization experiments. Bonferroni post hoc tests were used for group comparisons.
6.4 Results

In vitro screening of shRNA's constructs

We made reporter plasmids with D1R or D5R cDNA inserted in the 3' untranslated region (UTR) of a GFP expressing cassette (GFP-D1R or D5R-GFP). CHO cells were transfected with GFP-D1R alone (control), GFP-D1R co-transfected with shRNA’s (#1, #2, #3 or #4) and GFP-D1R co-transfected with shRNA designed to target D5R (this served as negative control). Reduction in GFP fluorescence intensity was used as an indicator of effective knockdown. This is due to the fact GFP is also degraded alongside the target sequence by the shRNA. Fluorescence intensity was measured at 24 (Fig. 6.1A) and 48 hours (Fig. 6.1B) after transfection respectively.

At 24 hours post transfection, all three shRNA constructs knocked down the reporter transcript significantly. One-way ANOVA revealed a significant main effect of knockdown in all 4 groups (Fig. 6.1A, 24 hours, $F_{1,5}=10.13$, *$p<0.05$). Bonferroni post hoc test revealed a significant difference in fluorescence intensity for each of the 4 constructs relative to GFP-D1R transcript alone (*$p<0.05$). At 48 hours, only shRNA construct #2 and #4 were able to maintain knockdown of reporter transcript (Fig. 6.1B, $F_{1,5}=5.249$, *$p<0.05$). No change in fluorescence intensity was observed when shRNA against D5R was co-transfected with GFP-D1R. This shows that D5R shRNA does not recognize the GFP-D1R transcript and specifically targets GFP-D5R transcript for degradation.
Figure 6.2(A and B) represents results from CHO cells transfected with GFP-D5R alone, shRNA (#1, #2, or #3) constructs co-transfected with GFP-D5R and an shRNA designed to target D1R co-transfected with GFP-D5R. Data was analyzed at 24 and 48 hours post transfection. 24 hours following transfection, all three shRNA constructs significantly knocked down the reporter transcript. One-way ANOVA revealed a significant main effect of knockdown in all 3 constructs (Fig. 6.2A, 24 hours, $F_{1,4}=12.72, *p<0.05$) and bonferroni post hoc test revealed a significant decrease in fluorescence intensity in each of the 3 constructs relative to GFP-D5R alone (*$p<0.05$).

At 48 hours, all three shRNA constructs continue to maintain knockdown of reporter transcript. (Fig. 6.2B, $F_{1,4}=20.93, *p<0.05$). Fluorescence intensity was unchanged in cells co-transfected with GFP-D5R and a shRNA against D1R. This suggests that shRNA (against D1R) does not target the GFP-D5R transcript for degradation, confirming its specificity.

**Quantitation of D1 and D5 receptors knockdown in the nucleus accumbens**

Mice were injected with either control (AAVshGFP) or D1shRNA (AAVD1shR) or D5shRNA (AAVD5shR) bilaterally into the nucleus accumbens. After a 3 week recovery period, RNA was extracted from injected tissue and quantitative PCR was performed. Beta-actin was used as within sample control and expression of genes was normalized to beta-actin. There was 60% reduction in D1R mRNA levels in animals injected with AAVD1shR compared to AAVshGFP injected animals (Fig. 6.3A,
No difference in D5R mRNA levels was observed in either AAVshGFP or AAVD1shR injected group. Similarly, animals injected with D5shRNA showed a 44% reduction in D5R mRNA expression relative to controls (Fig. 6.3B, \(F_{3,18}=5.05, *p<0.05\)), while D1R mRNA levels was unchanged. These results indicate that shRNA are specific for their intended targets and that a reasonable reduction of mRNA levels was achieved in the injected regions.

### Locomotor response to SKF81297 (D1-like agonist) and Cocaine

Locomotor response to saline and to D1-like agonist (SKF81297) at 5mg/kg was measured in animals injected with AAVshGFP, AAVD1shR or AAVD5shR. A two-way ANOVA was performed. Control mice injected with AAVshGFP exhibited significant locomotor stimulation by 5mg/kg SKF81297 (saline vs SKF81297, \(*p<0.01\)), while no locomotor stimulation was observed in mice with D1R knockdown (\(p=0.426\)). In contrast, mice with D5R knockdown retained locomotor stimulation by SKF81297 (\(*p<0.01\)).

The locomotor activity of AAVD1shR group following SKF81297 injection was significantly lower than both the AAVshGFP and AAVD5shR groups (#\(p<0.01\)). A main effect of drug (SKF81297) was observed (Fig 6.5A, \(F_{1,54}=25.149, *p<0.01\)), but no significant main effect of group (virus injection) (\(F_{2,54}=2.951, p=0.061\)) or group x drug (\(F_{2,54}=2.782, p=0.071\)). Locomotor activity in response to cocaine injection (10mg/kg) is shown in Fig. 6.5B. A two-way ANOVA revealed a main effect of dose (\(F_{1,94}=70.114, *p<0.01\)).
$p<0.01$) as well as a significant group x dose interaction $F_{2,94}=3.839$, $p<0.05$), but no main effect of group ($F_{2,94}=2.310$, $p=0.105$). A post hoc analysis show that all three groups showed significant locomotor stimulation when injected with 10mg/kg cocaine (*$p<0.01$) (saline vs cocaine). However, animals with D1R knockdown exhibited significantly lower locomotor activity compared to control and D5R knockdown animals (#$p<0.01$). Data are presented as mean±SEM.

**Conditioned Place Preference and Locomotor Sensitization**

In order to ascertain the role of D1 and D5 receptors in cocaine’s rewarding effects, conditioned place preference was performed on animals injected with AAVshGFP (n=19), AAVD1shR(n=23) or AAVD5shR (n=19). CPP score is defined as the time spent in drug paired chamber during post-test minus time spent in drug paired chamber during pre-test. A one-way ANOVA shows a significant main effect of group (Fig. 6.6A, $F_{2,59}=4.434$, $p<0.016$). Bonferroni post hoc analysis showed only a trend toward statistical significance in CPP score in animals with D1R knockdown ($p=0.06$). On the other hand, CPP score was unaffected in animals with D5R knockdown.

Locomotor sensitization was also measured during the CPP paradigm by recording locomotor activity during cocaine pairing days. Fig. 6.5B represents locomotor activity measured over 30minutes on the 1$^{st}$ (Day 1) and 4$^{th}$ (Day 4) cocaine pairing days. Repeated measures ANOVA were used to assess sensitization in all three groups. Animals with D1R knockdown did not exhibit cocaine induced locomotor sensitization.
(Day 1 vs Day 4, \( p=0.09 \)). Conversely, both control and D5R knockdown animals exhibited locomotor sensitization to cocaine (Day 1 vs Day 4, \(* p<0.01\)). Data are presented as mean±SEM.

**Cocaine's Chronic Effects**

In order to determine the role of D1 or D5R in mediating cocaine’s chronic effects, we injected animals with different doses of cocaine (2.5, 5 and 20mg/kg) following CPP paradigm. Locomotor activity was measured for 30 minutes. Data are presented as mean±SEM. In response to 1mg/kg cocaine, locomotor suppression was observed in controls and D5R knockdown animals. However, 1mg/kg cocaine did not produce any effect on animals with D1R knockdown. There was no main effect of group (Fig. 6.7A, \( F_{2,123}=1.833, p=0.164 \)). A significant effect of drug was observed (\( F_{1,123}=8.251, p<0.05 \)), but no group x drug (\( F_{2,124}=1.394, p=0.252 \)). Cocaine at 2.5mg/kg induced significant locomotor suppression in D1R knockdown animals. There was a main effect of group (Fig 6.7B, \( F_{2,118}=3.866, p<0.05 \)). A significant effect of drug was observed (\( F_{1,118}=10.084, p<0.05 \)), but no group x drug treatment (\( F_{2,118}=0.586, p=0.558 \)). However, neither suppression nor stimulation to cocaine (2.5mg/kg) was observed in animals with D5R knockdown. Post hoc tests (bonferroni) revealed a significant difference (*\( p<0.05 \)) between saline and cocaine (2.5mg/kg) in AAVshGFP and AAVD1shR, but not AAVD5shR (\( p=0.297 \)) group. When animals were injected with 5mg/kg cocaine, there was a main effect of group (Fig 6.7C, \( F_{2,118}=3.832, p=0.02 \)) and drug (\( F_{1,118}=12.182, p<0.01 \)).
Bonferroni post hoc tests revealed a significant locomotor stimulation in animals with D5R knockdown. (*$p<0.01$). On the other hand, no locomotor stimulation was observed in control or D1R knockdown animals (AAVshGFP, $p=0.212$, AAVD1shR, $p=0.283$). Finally, at 20mg/kg cocaine, a significant main effect of drug ($F_{1,54}=173.13$, $p<0.01$) but no main effect of group ($F_{2,54}=2.495$, $p=0.092$) was observed. However no significant interaction between group x drug was seen ($F_{2,54}=2.595$, $p=0.084$). Post hoc analysis revealed significantly higher locomotor stimulation in all three groups (*$p<0.01$).

6.4 Discussion

In this study we sought to investigate the contribution of Dopamine D1 and D5 receptors expressed in the NAc in cocaine’s rewarding and locomotor stimulating effects. Using adeno associated viral vectors expressing short hairpin RNA’s we modulated expression of these receptors in a brain region specific manner. There was no change in basal locomotion in either D1R or D5R knockdown animals. D1R knockdown group responded less to cocaine’s locomotor stimulating effects and also failed to locomotor sensitizing effects of cocaine. No change in conditioned place preference to cocaine was observed in the D5R knockdown group; however D1R knockdown group exhibited a trend toward lower conditioned place preference. The techniques employed in this study complement some of the early approaches used to investigate the contribution of D1 and
D5 receptors in mediating cocaine’s effects. However, it is unique in that it allows us to alter expression of either D1 or D5 receptors in specific brain regions. We did not observe any effect of D1R knockdown on basal locomotion. D1R knockout (KO) mice have been shown to have increased basal locomotor activity (Xu et al., 1994a, Xu et al., 1994b, Wong et al., 2003). The difference between the two studies is that we achieved partial knockdown of D1R in the NAc whereas the generated transgenic animals with complete ablation of the D1R gene. We found D1R knockdown animals did not respond to the locomotor stimulating effects of SKF81297. On the other hand, both control and D5R knockdown animals exhibited locomotor stimulation.

The results from D1R knockdown experiments are in agreement with other studies (Clifford et al., 1999, Centonze et al., 2003) that indicate a facilitatory role for D1 receptors in agonist induced locomotion, although this is first time anyone has showed that D1R’s within the NAc contribute to this effect. Results from D5R knockdown animals however, are in contrast to studies that showed reduced response to various doses of SKF81297 in D5R KO mice (Holmes et al., 2001, Elliot et al., 2003). Holmes et al. reported reduced locomotor stimulation by SKF81297 at low doses (1 and 2mg/kg), however at 3mg/kg, no difference in locomotor stimulation was observed between wild-type and D5R KO mice. We tested locomotor stimulating effects of SKF81297 at 5mg/kg. It is possible that at this dose the effect is mediated primarily by D1 receptors, as we observe D1R knockdown mice to have an attenuated response to SKF81297 at this dose. Unlike knockout studies where the locomotor response to cocaine is almost entirely

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abolished (Xu et al., 1994a), we see that D1R knockdown animals have reduced locomotor stimulation to cocaine compared to controls. As results from in vivo quantification of D1R show, knockdown is not a 100%, suggesting that any remaining D1R within the nucleus accumbens could transduce signal to produce a response to cocaine’s locomotor stimulating effects. On the other hand, no change in cocaine induced locomotor activity was observed in D5shRNA injected animals.

Overall, these results suggest that locomotor stimulating effect of SKF81297 and cocaine are mediated predominantly by D1R in the NAc. Injected animals were also tested for cocaine’s rewarding effects. The question we asked was if D1R and D5R in the nucleus accumbens (without distinguishing the shell and core subregions) had any role in mediating cocaine’s rewarding effects. The shRNA knockdown of D5R had no effect on cocaine CPP scores. In contrast, we found a trend toward reduced cocaine CPP score in D1R knockdown animals. There was a considerable reduction but scores did not reach statistical significance (p = 0.067). However, CPP score of D1shRNA injected animals was significantly lower than that of D5shRNA injected animals (*p< 0.01).

One possibility for variation in our results could be attributed to inconsistency in injection spread. NAc consists of two distinct anatomical subdivisions namely the shell and core. While NAc shell received inputs from the subiculum and basolateral amygdala (BLA), the core is innervated by inputs from parahippocampal regions and BLA (Groenewegen et al., 1999, French and Totterdell, 2003). These two regions are also known to have divergent roles in mediating reward-related behaviors. For example,
cocaine injections directly into the shell and not core produced CPP (Liao et al., 2000) and lesions of medial core and not shell with 6-hydroxydopamine inhibited CPP, indicating these regions may have opposing roles in mediating reward (Sellings and Clarke, 2006). The injections were intended to target NAc as a whole trying to cover as much of two anatomical regions as possible. It is possible that variability in CPP score could have resulted from variable distribution of the virus between these two regions.

The conditioned place preference experiment was performed using a modified protocol. This protocol allows us to measure locomotor sensitization simultaneously (Shimosato and Ohkuma, 2000). Locomotor sensitization is a phenomenon that results from repeated exposure to drugs of abuse. This phenomenon manifests as a progressive increase in response to motor effects of the drug. Underlying this are neuroadaptations that could ultimately predispose an individual to relapse. Evidence that indicate a role for dopamine D1-like receptors in mediating locomotor sensitization include one where cocaine sensitization increased following intra-accumbal injections of a D1R and D5R agonist (SKF-82958) (De Vries et al., 1998). A recent study indicated a possible role for D1-like receptors in mediating changes in spine morphology in the nucleus accumbens following repeated cocaine exposure (Ren et al., 2010). NAc is known to undergo widespread neuroplasticity following repeated cocaine injections (Vanderschuren and Kalivas, 2000). Most of these changes manifest as increases in spine density on nucleus accumbens core medium spiny neurons (Li et al., 2004). Our own lab has shown that cocaine induced changes in spine density within the nucleus accumbens shell requires
dopamine transporter inhibition (Martin et al., 2011). Although there is evidence to suggest that spine density changes occur in both the NAc shell and core, we did not pursue one over the other. We wanted to test which one of the two receptors in the NAc contributed to locomotor sensitization.

We found reducing the expression of D1R in the accumbens inhibited development of locomotor sensitization. No change in locomotor sensitization was observed in D5shRNA injected animals. These results indicate a role for D1R in the nucleus accumbens in mediating cocaine’s locomotor sensitizing effects. This is for the first time anyone has shown that D1R and not D5R underlie locomotor sensitizing effects of cocaine. Fact that we see locomotor sensitization in D5R injected animals effectively rules out its role in sensitization. However it is important to keep in mind that D5R are expressed in low numbers in the nucleus accumbens relative to D1R.

We followed up the modified CPP paradigm by testing the animal’s response to varying doses of cocaine. This experiment was performed a week following CPP experiment. These sets of experiments were done to test the effect of sensitization or lack thereof on dose response to cocaine. Cocaine at low doses is known to produces locomotor suppression (George, 1989, 1990). We find that at 1mg/kg cocaine, control and D5shRNA injected animals have reduced locomotor activity relative to saline, however D1shRNA injected mice do not respond to cocaine’s locomotor suppressing effects. At a slightly higher dose of 2.5mg/kg, control and D1shRNA injected animals responded to cocaine’s suppressing effects; however D5shRNA injected animals did not.
At 5mg/kg cocaine, control and D1shRNA exhibited neither suppression nor stimulation, but D5shRNA injected animals showed an increase locomotor activity relative to saline. All three groups exhibited increased locomotor activity at 20mg/kg cocaine, although the magnitude of stimulation was significantly higher in D1shRNA injected animals relative to saline. Taken together, these results indicate a shift in the dose response curve to the right in D1shRNA injected animals whereas a shift to the left is observed in the D5shRNA injected animals.

An important caveat to consider while interpreting these results is the underlying effect of sensitization. However, both control and D5shRNA injected animals sensitized to cocaine but respond differently to various doses of cocaine. It appears that D5R plays a regulatory role in controlling locomotor behavior at low doses of cocaine. Locomotor suppression seen in control animals at low doses could in part be due to activation of D5R. The reduction in D5R expression in the D5shRNA injected group reduces the influence D5R on locomotor behavior thus shifting the dose response curve to the left. An inhibitory role for D5R in locomotor activity was first proposed by Dziewczapolski et al. They reported an increase in locomotor activity induced by a D1-like agonist in animals injected with an antisense oligonucleotide against D5R (Dziewczapolski et al., 1998). It has also been shown that D1R knockout mice failed to induce long term potentiation in the striatal neurons whereas pharmacological blockade of D5R prevented long term depression (Centonze et al., 2003), further providing evidence for divergent roles for these receptors.
In summary, we used short hairpin RNA’s to knockdown the expression of D1R or D5R selectively in the nucleus accumbens in order to differentiate roles of these receptors in mediating cocaine’s effects. We found that D1R in the nucleus accumbens are important for mediating cocaine’s acute and chronic effects, although its role in cocaine’s rewarding effects remains inconclusive. D5R, on the other hand, may have an inhibitory role in mediating cocaine induced locomotion at low doses.
Figure 6.1 In vitro knockdown of GFP-D1R transcript (A) Four different D1shRNA constructs were co-transfected with a target plasmid (GFP-D1R) into CHO cells. Fluorescence intensities, representing GFP-D1R expressing levels, were measured using ImageJ software. All 4 constructs knocked down their target transcript significantly (*p<0.05). (B) At 48 hour time point, constructs #2 and #4 maintained significant knockdown of target transcript (*p<0.05). No reduction in fluorescence intensity was observed when shRNA against D5R was co-transfected with target plasmid (GFP-D1R) at 24 and 48 hours.
Figure 6.2 *In vitro* knockdown of GFP-D5R transcript. (A) Three different D5shRNA constructs were co-transfected with a target plasmid (GFP-D5R) into CHO cells. Fluorescence intensities representing GFP-D5R expressing were measured using ImageJ software. All 3 constructs knockdown target transcript (GFP-D5R) in a significant manner (*p* < 0.05) (B) At 48 hours, the three constructs maintained knockdown of their target transcript (*p* < 0.05). No reduction in fluorescence intensity was observed when shRNA against D1R was co-transfected with target transcript (GFP-D5R) at 24 and 48 hours.
Figure 6.3 Quantitation of D1R or D5R knockdown *in vivo*. Three groups of mice were injected with AAVshGFP, AAVD1shR, and AAVD5shR in the NAc. Three weeks after injection, D1R and D5R mRNA levels at NAc were determined relative to beta actin. (A) There was a 60% reduction in D1R mRNA levels in AAVD1shR injected animals relative to AAVshGFP injected animals (*p=0.05). D5R mRNA levels was unchanged in AAVshGFP and AAVD1shR injected animals (B) Mice injected with AAVD5shR showed a 44% decrease in D5R mRNA levels relative to AAVshGFP group (*p=0.05). D1R receptor expression was unaffected in either group.
Figure 6.4 DAB staining of perfused tissue. Animal was injected with either AAVD1shR (left) or AAVD5shR (right) bilaterally into the nucleus accumbens. The AAV viruses express the turboRFP and shRNA construct in the same transcript. DAB staining (for turboRFP) shows the areas of virus infection and shRNA expression in the nucleus accumbens.
Figure 6.5 D1-like agonist and Cocaine induced locomotor activity in AAVGFP, AAVD1shR or AAVD5shR groups. (A) Mice injected with AAVshGFP or AAVD5shR showed locomotor stimulation following i.p injection of SKF81297 (*p<0.05). D1R knockdown did not show locomotor stimulation by SKF81297, they also had reduced locomotor activity relative to control AAVshGFP and AAVD5shR injected animals (#p<0.01). (B) Cocaine induced locomotor activity at 10mg/kg. Cocaine induced locomotor stimulation was observed in all three groups (sal vs coc, *p<0.05). There was a significant decrease in locomotor activity after cocaine injection in AAVD1shR group relative to AAVshGFP and AAVD5shR (#p<0.01)
Figure 6.6 Conditioned place preference and locomotor sensitization in AAVshGFP, AAVD1shR and AAVD5shR injected animals. (A) CPP score is defined as time spent in drug paired chamber in post-test minus time spent in drug paired chamber in pre-test. All 3 groups displayed significant CPP induced by cocaine at 10mg/kg. AAVD1shR injected animals showed a trend toward reduced CPP score when compared to AAVshGFP group ($p=0.067$) and the CPP score is significantly lower compared to AAVD5shR injected animals (#$p<0.01$). (B) Locomotor sensitization in AAVshGFP, AAVD1shR and AAVD5shR injected animals. AAVD1shR injected animals did not sensitize to cocaine (Day 1 vs Day 4, *$p<0.05$). The magnitude of sensitization was lower in AAVD1shR injected animals relative to control and AAVD5shR injected animals ($^\delta p<0.05$).
Figure 6.7 Cocaine induced locomotor activity was assessed in animals injected with AAVshGFP, AAVD1shR or AAVD5shR in the nucleus accumbens. (A-D) Locomotor activity following administration of 1, 2.5, 5.0 and 20mg/kg cocaine. (A) At 1mg/kg, locomotor suppression was observed in AAVshGFP and AAVD5shR groups (sal vs coc, *p<0.05). (B) At 2.5mg/kg, locomotor activity was significantly reduced in AAVshGFP and AAVD1shR groups (sal vs coc, *p<0.05). (C) At 5mg/kg, locomotor activity was significantly increased in AAVD5shR group (sal vs coc, *p<0.05). (D) A significant increase in locomotor activity was observed in all three groups (sal vs coc, *p<0.05).
In this dissertation, we set out to identify environmental and genetic factors that may contribute to the etiology of autism spectrum disorders. We tested the effects of environmental factors such as diesel exhaust particles and food additives on fetal brain development. Diesel exhaust particles when exposed during pregnancy increased locomotor activity and rearing behaviors in the offspring. It also caused an increase in grooming behaviors, although social behaviors were unaffected. We also tested the effects of prenatal exposure to food additives on mouse social behaviors. We found that feeding pregnant animals with food colors reduced social interaction in the offspring. We also characterized a PTPRT knockin mouse line and showed that these animals exhibited increased social interaction behaviors. And finally, we showed that D1R within the nucleus accumbens are critical for mediating cocaine induced locomotor activity and locomotor sensitization.

Adverse effects of diesel exhaust particles on pulmonary and cardiovascular function are well established. We hypothesized that exposure during pregnancy might adversely affect fetal brain development and induce autism-like behavior in offspring. Autism is diagnosed in children around the age of 2. Early diagnosis relies on the parent’s ability to identify delays in developmental milestones as well as clinical evaluation by
healthcare practitioners. As of now, there are no biomarkers that can be tested for and diagnosis relies almost exclusively on clinical observations of behavior. Similarly, screening for autism-like behavior in mouse models depends on the use of standardized behavioral assays. We employed different behavioral methods to screen for deficits in social communication, social interaction and repetitive and stereotyped behaviors, the three core symptoms of autism. Offspring of DEP exposed animals exhibited no deficits in social behaviors however displayed increased grooming and rearing behaviors. These findings indicate that fetus is vulnerable to environmental insults especially during critical periods of development. Diesel exhaust particle is one such factor that can negatively affect neural pathways involved in mediating repetitive and stereotyped behaviors. Lack of deficits in different measures of social behavior indicates that these behaviors may be resistant to DEP exposure.

There are several limitations to this study. First, it is essential to note that animals groom and rear for a variety of reasons and these behaviors may not necessarily be induced by DEP exposure per se. Furthermore, repetitive behaviors can be simple motor stereotyped behaviors or may manifest as a result of a higher order cognitive deficit. It would be important to separate these two behaviors. One way to address this question would be test animals for deficits in impulsive behavior using a 5 choice serial reaction time task. This would help clarify if repetitive behaviors observed in our animals are simple manifestations of lower order stereotyped behaviors or a result of attentional impairments. Another limitation of this study is the dose of DEP used. Doses employed
in this study are higher than what an average human would inhale in his lifetime. Given our observations, it is possible that at normal doses we might not see an effect. However, the rationale (for selecting this dose) was if behavioral deficits were observed at higher exposures, there is a possibility that low dose DEP exposure in combination with genetic factors could predispose individuals to develop ASD-like phenotype. Future experiments could be directed toward understanding molecular mechanisms underlying behavioral deficits observed in our animals. Subchronic DEP exposure is known to induce changes in immune function via activation of TNFα in brain regions such as the midbrain (Levesque et al., 2011). There is a possibility that acute (pregnancy through gestation) exposures could elicit an immune response in the offspring. This can be confirmed by measuring levels of pro-inflammatory markers such IL-1 and TNFα in the offspring. Another possibility is activation of immune response in the mother consequently putting the fetus at risk. It is known that maternal infection is a risk factor for autism (Shi et al., 2003). This can be answered by looking at levels of inflammatory cytokines such as IL-6, which have been previously shown to be critical for mediating behavioral changes in offspring (Smith et al., 2007). Another approach would be to combine DEP exposure with simultaneous activation of IL-6 to see if the effects are additive and capable of inducing ASD-like phenotype in the offspring.

In chapter 3 we present data from a preliminary study testing the role of food additives as a possible environmental factor in the etiology of autism. We wanted to test if treating pregnant mice with a diet consisting of high concentrations of food additives
induced autism-like behavior in the offspring. We compiled a list of 20 food additives based on human consumption data. We placed them in 4 different groups and fed pregnant mice with these additives from gestation through weaning. Results from this pilot study indicated that certain food additives when given at high concentrations during pregnancy can induce behavioral changes in offspring. Given the preliminary nature of these findings, it is essential to replicate initial observations by repeating these experiments in different cohorts of mice. Also, behavioral deficits observed (in the form of reduced social interaction) are from using a combination of five different food additives. We do not know if the observed effect is due to a single compound or an additive effect induced by a combination of all 5 additives. In order to narrow down the list and identify compounds that might contribute to the observed effects we plan to test individual compounds at high doses. A downside to this approach is the amount of time required to test different combinations of food additives. Once compounds are identified we intend to study their metabolism. Once a compound or a combination of compounds is identified we could take a genomics approach and identify genes that are involved in their metabolism. We could then create transgenic lines of those genes and test to see if the combination of genetic and environmental factors increases the odds of inducing an autism-like phenotype.

In chapter 3, we characterized a protein tyrosine phosphatase (PTPRT) knock in mouse line. We generated a knock-in mouse model of PTPRT, a transmembrane receptor. We found that inactivating the phosphatase domain of PTPRT protein did not induce
anxiety-like behaviors. Ironically, we found PTPRT knockin mice to have increased social interaction relative to wild-type animals. It would of interest to know if inactivation of PTPRT function affected other social behaviors such as social communication. This can be tested in our mice by performing additional behavioral experiments such as social transmission of food preference and ultrasonic vocalizations behaviors. We speculated that inactivating the phosphatase function of PTPRT affected phosphorylation/dephosphorylation states of proteins critical for synapse formation resulting in altered neural pathways and animal behaviors relevant to ASD. This can be tested by measuring phosphorylation states of proteins such as STAT3 and paxillin, known substrates of PTPRT. There are several limitations to study. We know from literature that mutating aspartate to alanine inactivates the phosphatase function of the protein. However, this remains to be tested in our transgenic line. There is a possibility that inactivation of the phosphatase domain might not have affected the function of the protein or the lack of phosphatase function could have been compensated by other members of the tyrosine phosphatase family.

In the final part of the dissertation, we used short hairpin RNA’s to help distinguish dopamine D1 from D5 receptors in mediating cocaine’s rewarding and locomotor stimulating effects. We found that D1R knockdown within the nucleus accumbens attenuated locomotor stimulation induced by cocaine. We did not observe any changes in cocaine induced locomotor stimulation in D5R knockdown animals. These results indicate that locomotor stimulating effects of cocaine are mediated primarily by
D1R. Although studies using knockout models and pharmacological inhibitors have long suggested a role for D1R's in cocaine induced locomotor activity with the use of this model we were able separate its role from D5R, due to specificity of the shRNA. We were also able to identify specific anatomical regions (expressing D1R) that may contribute to this effect. However, D5R are expressed in low numbers in the nucleus accumbens and this may preclude us from ruling out its involvement in mediating cocaine’s effects. We also showed that D1R knockdown animals had an attenuated response to cocaine induced reward behavior. It is important to note that the findings were not statistically significant partially due to variability of response to cocaine’s rewarding properties in the D1R knockdown animals. We believe the variation may in part be due to variable spread of the virus containing the shRNA in the nucleus accumbens. One possible way to address this would be inject the shRNA in either the shell or the core regions of the nucleus accumbens.

We also showed that D1R within the nucleus accumbens may mediate cocaine induced locomotor sensitization. These findings suggest that D1R mediate some of the long lasting effects of cocaine and may play a critical role in drug seeking behavior following relapse. And finally, we observed that D5R knockdown are hypersensitive to cocaine’s locomotor effects at low doses. These findings indicate that cocaine’s locomotor effects are mediating primarily by D1R however at low doses D5R may have an inhibitory role in mediating cocaine’s locomotor effects. Very little is known about the function of D5R in mediating cocaine’s locomotor and rewarding effects. Previous
studies attempting to study D5 receptors using knockout models have showed little evidence for D5R’s role in mediating any of cocaine’s effects. Using short hairpin RNA’s allowed us to lessen the effect of compensatory adaptations that may have resulted in knockout models. A major limitation of using shRNA’s, however, is the inability to achieve a 100% knockdown gene expression. There is also a possibility of reduced shRNA efficiency over a period of time which would effectively limit us from studying chronic effects of cocaine exposure. Multiple brain regions are involved in mediating cocaine’s effect. This model allowed us to test the contribution of dopamine receptors in different brain regions within the brain reward pathway. Future studies could involve investigating the function of D1R expressed in other brain regions such as dorsal striatum in mediating cocaine’s effects.

Work related to two different disease conditions were discussed in this dissertation: cocaine addiction and autism spectrum disorders. Besides being conditions of the central nervous system, a possible common feature between these disorders is a dysfunctional dopamine system. A recent study identified a de novo mutation in the dopamine transporter gene in a single autistic patient. When the mutation was introduced into flies, they exhibited hyperlocomotion (Hamilton et al., 2013). Similarly, specific behavioral phenotypes associated with ASD such as repetitive behaviors have been linked with polymorphisms in dopamine receptors including D1R, D3R (Hettinger et al., 2008, Staal et al., 2012). Drugs such as risperidone a dopamine receptor antagonist have been prescribed to alleviate irritability and reduce stereotyped behaviors in children
diagnosed with ASD (McPheeters et al., 2011). We hadn’t investigated changes in dopamine system in our autism mouse models; however future studies could be aimed exploring this aspect.
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